

AD_____

Award Number: DAMD17-98-1-8269

TITLE: Analysis BAP-1 as a Ubiquitin Hydrolase in the BRCA1
Pathway

PRINCIPAL INVESTIGATOR: David C. Schultz, Ph.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, Pennsylvania 19104

REPORT DATE: October 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020909 058

Table of Contents

Cover.....	1
SF 298.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	9
References.....	10
Appendices.....	N/A

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 2001	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Oct 00 - 30 Sep 01)	
4. TITLE AND SUBTITLE Analysis BAP-1 as a Ubiquitin Hydrolase in the BRCA1 Pathway			5. FUNDING NUMBERS DAMD17-98-1-8269	
6. AUTHOR(S) David C. Schultz, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Wistar Institute Philadelphia, Pennsylvania 19104 E-Mail: dshultz@wistar.upenn.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The BRCA1-associated protein ZBRK1 is a member of the KRAB domain-zinc-finger protein (ZFPs) superfamily. The more than 220 KRAB-ZFPs encoded by the human genome function as gene-specific silencers. Silencing requires binding to the co-repressor, KAP-1, that in turn coordinates the activities of large macromolecular complexes that modify chromatin structure. The PHD finger and bromodomain of KAP-1 recruits the NuRD HDAC complex whereas a separate region of KAP-1 binds directly to the chromoshadow domain of the HP1 protein family. Since the HP1 chromodomain can bind to the methylated Lys-9 in histone H3, we looked for this activity in KAP-1 repression complexes. We have recently discovered a novel KAP-1 associated H3, Lys-9 specific histone methyltransferase, SETDB1. The enzymatic activity of SETDB1 increased HP1 binding to histone H3 and CHIP experiments showed co-localization of KAP-1, SETDB1, and HP1 along with increased H3 Lys-9 methylation of chromatin at an endogenous target gene to stably repress expression. KAP-1 is the first example of a co-repressor/scaffold protein that can coordinate the sequential recruitment of HDAC complexes, histone methylase, and the deposition of HP1 at a euchromatic locus to silence gene expression in a manner consistent with epigenetic regulation. These studies define the molecular machinery utilized by ZBRK1 to regulate gene expression, current studies are focused at evaluating the role of KAP1:SETDB1:HP1 and ZBRK1:BRCA1 regulation of endogenous target genes.				
14. SUBJECT TERMS Breast Cancer, Epigenetic regulation, Histone methylation, HP1, KRAB-ZFP				15. NUMBER OF PAGES 43
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Introduction:

The molecular basis of breast cancer is rapidly being defined. Genetic analyses of large affected kindreds have identified deleterious mutations in at least three to five genes (i.e. BRCA1, BRCA2, TP53, CHK2, and PTEN) that confer inherited susceptibility to breast cancer. Although mutations in the genetic code of these genes explains the molecular anatomy of hereditary breast cancers, little is known about the molecular genetics of sporadic forms of the disease. Although well-defined genetic mutations in tumor suppressor genes and oncogenes firmly establishes a causative role for the effected gene during pathogenesis, a frenzy of studies have revealed additional mechanisms that contribute to oncogenic conversion, including epigenetic changes in regulation of gene expression. A plethora of biochemical data is pointing to DNA-packaging proteins as the key regulators of epigenetic controlled expression or silencing of the genetic code. Distinct post-translational modifications of the NH₃-terminal tails of the core histone proteins can generate synergistic or antagonistic interaction affinities for non-histone chromosomal proteins that in turn dictate the dynamic transitions between transcriptionally active or silent chromatin states. It is the combinatorial nature of this developing "histone code" that may considerably extend the information potential of our DNA. It is postulated that this epigenetic marking system represents a fundamental regulatory system that will impact nearly all chromatin-templated processes, with global consequences observed in cell-fate decisions in both normal and pathological development. An increasing number of reports describe oncogenic mutations in the molecular machinery responsible for maintaining chromatin structure, further emphasizing the importance of chromatin in regulation of transcription and genome stability during tumorigenesis (1).

The **research focus** during the final year of this fellowship continued to address specific structural and biochemical questions about mechanisms of transcriptional repression conferred by the KRAB domain zinc finger protein superfamily of sequence specific transcription factors. These studies are particularly relevant to breast cancer research in several ways. First, The BRCA1-associated protein ZBRK1 is a member of the KRAB domain-zinc-finger protein (ZFP) family (12). This association implies a role for the KRAB:KAP-1 repression system in BRCA-mediated transcription responses. Second, the expression of HP1 α , a non-histone chromosomal protein, has been shown to be significantly down regulated in breast cancer cells that acquire a more aggressive metastatic phenotype (4). We have previously shown that KAP-1 directly interacts with the HP1 family of proteins. Furthermore, during the support period of this award I biochemically purified a macromolecular complex that implicates HP1 in replication-dependent maintenance of heterochromatin. We postulate that loss of HP1 α expression results in either activation of pro-metastasis genes or global chromosomal instabilities. Finally, recent studies have demonstrated differential programs of gene expression during malignant breast cancer progression that correlate with tumor stage and clinical outcomes (11). Profiling gene expression in breast tumors may assist molecular diagnosis, determine therapeutic regimens, and predict patient survival. Thus, once basic mechanisms of transcription regulation in chromatin are defined, new and innovative approaches, both genetic and pharmacologic, can be designed to reverse aberrant, gene expression profiles involved in cancer phenotype. I believe the findings of this research will have broad implication in many scientific disciplines, including transcriptional regulation, structural biology, chromatin structure/function, development, and cancer biology.

Summary of Research

The KRAB (Krüppel Associated Box) domain is one example of an abundant amino acid sequence motif found at the NH₂-terminus of 240 independent Krüppel/TFIIIA type C2H2 zinc finger proteins. This highly conserved domain displays potent, DNA-binding dependent repression of transcription that requires a unique corepressor protein, KAP-1(3, 5). Structurally, KAP-1 possesses consensus amino acid sequences for a RING finger, B-boxes, leucine zipper Coiled-Coil region, PHD finger, and bromodomain. Biochemical studies of KAP-1 indicate that the tripartite RBCC region functions as an integrated structural unit that is necessary and sufficient for KAP-1 oligomerization and KRAB recognition (6, 7). During the support period of this fellowship, amino acid sequences carboxy-terminal to the RBCC domain were dissected into at least two non-overlapping repression domains. The first of these domains is defined by amino acids that facilitate a direct interaction between KAP-1 and mammalian members of the non-histone chromosomal protein, heterochromatin protein 1 (HP1). Mutations within a core pentapeptide sequence within KAP-1 (PxVxL) disrupt this interaction *in vitro* and reduce the overall efficiency of KAP-1 to repress transcription when tethered to DNA templates *in vivo*. Immuno-localization studies in interphase nuclei of NIH/3T3 cells revealed that KAP-1 staining is almost exclusively nuclear, excluded from nucleoli, and concentrated in islands of condensed DNA consistent with pericentromeric heterochromatin. Dual staining immunofluorescence studies revealed that endogenous KAP-1 colocalized with both heterochromatic and euchromatic HP1 proteins in interphase nuclei. From these data, we postulated that gene-specific repression by the KRAB-ZFP/KAP-1 complex in part may be a consequence of the formation and/or maintenance of heterochromatin-like chromosomal environments (9).

The KAP-1 PHD finger and bromodomain define a separate transcriptional repression activity. This tandem arrangement of motifs is frequently observed in transcriptional cofactor proteins, but functionally remains to be fully defined. I have shown that the PHD finger and bromodomain of KAP-1 form a cooperative unit that is required for the optimal transcriptional repression properties of KAP-1. As an independent strategy to gain some insights to the biochemical function of the PHD finger, we have determined the first three-dimensional structure of the minimal PHD finger of KAP-1 by NMR methods. Our studies revealed that the PHD domain from the KAP-1 corepressor binds zinc in a cross-brace topology between anti-parallel β -strands reminiscent of RING domains, suggesting a potential role in protein:protein interactions (2). Substitution of highly related PHD fingers or bromodomains failed to restore repression activity, suggesting high specificity in their cooperative function. Moreover, single amino acid substitutions in either the bromodomain or PHD finger, including ones that mimic disease-causing mutations in the hATR_X PHD finger abolish repression. These data are consistent with this bipartite domain providing a specific interface for protein-protein interactions. A search for effectors of this repression function identified an interaction with a unique isoform of the Mi-2 α protein, an integral component of the histone deacetylase complex/chromatin remodelling complex NuRD. Endogenous KAP-1 is associated with Mi-2 α and other components of NuRD, and this *in vivo* association requires the PHD finger and bromodomain of KAP-1. Furthermore, I demonstrated that KAP-1 mediated silencing *in vivo* requires association with NuRD and HDAC activity. These data suggest the KRAB-ZFP superfamily of repressors functions to target the histone deacetylase and chromatin remodeling activities of the NuRD complex to specific gene promoters *in vivo* (10).

In the same two-hybrid screen that identified Mi-2 α as an effector of the KAP-1 PHD/bromodomain repression, an additional interacting protein was identified whose primary amino acid sequence revealed the presence of a SET domain, another conserved amino acid motif found in many proteins with roles in the regulation of chromatin structure. I have subsequently named this protein KIS1 for KAP-1 Interacting SET protein-1. Proteins that possess the SET domain homology

have been shown to possess intrinsic histone methyltransferase (HMTase) activity (8). To test whether KIS1 possessed HMTase activity similar to its related family members, I expressed and purified a recombinant GST-KIS1 fusion protein encoding for the entire putative catalytic domain. Unlike other SET domain proteins, the recombinant KIS1 protein failed to demonstrate any appreciable methylase activity towards a core histone substrate. From this observation, I postulated that KIS1 may require some post-translational modification or cellular cofactor in order to function as a histone methylase. Therefore, I generated a FLAG-epitope tagged mammalian expression vector. Peptide eluates of anti-FLAG immunopurified KIS1 from transiently transfected HEK293 cells revealed a dose-dependent histone H3-specific methyltransferase activity for both a core histone substrate and mononucleosomes. A partial deletion of the SET domain or synthetic amino acid substitutions of highly conserved residues in the SET domain significantly effect this enzymatic activity, whereas a deletion of the putative KID (KAP-1 Interaction Domain) domain had no effect. Thus, similar to other members of the SET domain family of histone methyltransferases, KIS1 requires the SET domain for methylase activity.

To confirm the specificity of the observed enzymatic activity for histone H3 and the specific site of methylation, I used purified recombinant GST-histone tail proteins as substrates in the methylase assay. These experiments confirmed that KIS1 is a histone H3 specific methyltransferase. This methylation event absolutely requires lysine-9 of histone H3, as a substrate possessing an arginine substitution of lysine-9 (GST-K4R) in the context where the remaining amino acids represent the wild-type histone H3 tail failed to be methylated by KIS1. Moreover, this observation indicates that additional post-translational modifications (i.e. acetylation, phosphorylation) of the substrate are not required for the activity of KIS1 for Lys 9 of histone H3. Methylation of an unmodified histone H3 tail by KIS1 increased the affinity of HP1 α for the NH3-terminal tail of histone H3. This binding of HP1 α to the H3 tail depends on the integrity of the chromodomain of the HP1 proteins, as a specific mutation (V21M) in this domain disrupts the binding activity. Interestingly, a mutation in the chromoshadow domain that effects the dimerization (I165K) of HP1 also impaired the HP1:histone interaction.

Since KIS1 is fully capable of methylating an unmodified histone H3, I tested whether other post-translational modifications (i.e. acetylation, methylation, and phosphorylation) of histone H3 effect the KIS1 methylase activity. For these assays a panel of peptide possessing either an individual or a combination of modifications were used as substrates. KIS1 robustly methylated the unmodified H3 substrate, but not the H4 peptide. As expected, methylation or acetylation of lysine 9 inhibited the methylation of the substrate. Phosphorylation of serine 10 also dramatically effected the methylation of the substrate. Furthermore, a substrate acetylated at lysine 14 was methylated by KIS1, albeit to a much lesser extent than the unmodified. These observations are quite similar to that previously observed for the related lysine 9 specific histone H3 methyltransferase SUV39H1, indicating that these proteins likely recognize the substrate in a similar fashion and possess a similar catalytic mechanism. Thus, I conclude that *in vivo* the ability of KIS1 to methylate histones within a target locus will likely require coordination with deacetylase complexes and putative histone phosphatases. Consistent with this hypothesis, pretreatment of calf thymus histones with the histone deacetylase complex NuRD significantly enhanced KIS1-mediated methylation of the substrate.

To confirm that endogenous KIS1 possesses methylase activity we derived monoclonal and polyclonal antibodies. Using polyclonal antisera against KIS1, I could efficiently immunodeplete nearly all the histone H3 methylase activity from a partially purified nuclear extract. Moreover, the pellet of the KIS1 immunoprecipitates retained a strong histone H3 activity. These observations

strongly suggest that KIS1 represents an abundant histone H3 methyltransferase *in vivo*. Indirect immunofluorescence staining of asynchronous populations of NIH/3T3 cells with both the monoclonal and polyclonal antibodies revealed that KIS1 is ubiquitously localized throughout the nucleoplasm, excluded from the nucleoli, and sometimes observed as aggregates in interphase nuclei. The apparent euchromatic staining patterns for KIS1, suggest that lysine-9 methylation by KIS1 in euchromatic regions of the nucleus likely facilitates transcriptional repression by recruiting HP1 to create localized heterochromatin environments. To test this hypothesis, I used a series of chromatin immunoprecipitations (CHIP) to evaluate the role of KIS1, lysine-9 methylation, and HP1 in the repression of an endogenous transgene regulated by the KRAB:KAP-1 repression system. These CHIP experiments revealed co-localization of KAP-1, KIS1, and HP1 along with increased H3 lysine-9 methylation in chromatin of the endogenous, euchromatic transgene that is stably silenced. The combination of these data indicates that KAP-1 functions as a corepressor/scaffold protein that coordinates the recruitment of HDAC complexes, histone methylases, and the deposition of HP1 proteins at a euchromatic locus to silence gene expression in a manner consistent with epigenetic regulation. A manuscript describing these summarized results is currently being prepared for submission to *Science* or *Cell*.

In an independent set of experiments, I designed a biochemical strategy to define new functions for the HP1 family of proteins. The rationale for these studies stems from an observation that steady state levels of HP1 α are significantly reduced in aggressive, metastatic breast carcinoma cell lines (4). This observation suggested that an imbalance in HP1-mediated nuclear functions is compromised during the metastatic transition. To date the functions of the HP1 protein family have been defined by genetics, cytology, and the protein partners that directly associate with the chromoshadow domain, including KAP-1, SP100, LBR, ATRX, and CAF1. In order to identify novel functions of the HP1 proteins, I have derived and characterized monoclonal antibodies specific to isoforms of the HP1 protein family that represent both heterochromatic (HP1 α) and euchromatic (HP1 γ). During biochemical fractionations of nuclear extract, I specifically found that HP1 α stably copurifies as a core component of a novel four-subunit protein complex of the DNA replication dependent chromatin assembly factor, CAF1. Conventional chromatography approaches revealed that these four subunits perfectly co-elute as a 1.5 MDa complex. Although this purified complex exclusively contained HP1 α , I was able demonstrate that CAF1 subunits may also associate with HP1 γ , albeit to a lesser extent. Chromatin immunoprecipitation experiments are revealing a very tight co-localization of the CAF1 subunits with HP1 α globally in an extended array of chromatin. These data suggest that the stable association of HP1 with CAF1 implicates a role for DNA replication dependent chromatin assembly in maintaining heritable states of chromatin structure. Thus, one hypothesis is that down regulation of HP1 α expression during metastatic conversion leads to global alterations in chromatin structure, in particular the activation of genes that contribute to the metastatic phenotype which are not normally repressed. Future experiments, including cDNA microarray, could be designed to identify sets of genes regulated by HP1 α , that may be responsible for the cellular phenotype. These data are also being prepared for submission to a journal that remains to be determined at this time.

Key research accomplishments

- Described targeting of the Histone Deacetylase Complex via KRAB-Zinc Finger Proteins: The PHD and Bromodomains of KAP-1 form a Cooperative Repression Domain that Recruits the Mi-2 α Subunit of the NuRD complex.
- Solved the first solution structure of the KAP-1 PHD finger.
- Identified an interaction between KAP-1 and KIS1, a novel histone H3, Lys-9 specific histone methyltransferase.
- Extensively characterized the enzymatic activity of KIS1.
- Demonstrated the colocalization of KAP-1, KIS1, and HP1 with an increase in histone H3, Lysine-9 methylation in chromatin of an endogenous gene regulated by the KRAB-ZFP:KAP-1 repression system.
- Biochemically purified a core 4 subunit complex containing HP1a and the Chromatin Assembly Factor (HP-CAF)

Reportable Outcomes:

Publications

Capili AD, **Schultz DC***, Rauscher III FJ, Borden KLB. Solution Structure Of The PHD Domain From The KAP-1 Corepressor: Structural Determinants For PHD, RING, And LIM Zinc-Binding Domains. **EMBO Journal**, 20: 165-177 (2001).

Schultz DC, Friedman JR, Rauscher III FJ. Targeting Histone Deacetylase Complexes Via KRAB-Zinc Finger Proteins: The PHD And Bromodomains Of KAP-1 Form A Cooperative Unit That Recruits A Novel Isoform Of The Mi-2 α Subunit Of NuRD. **Genes and Development**, 15: 428-443 (2001).

* Indicates that author should be considered an equal contributor.

Manuscripts

Rauscher III FJ, Jensen DE, Patel G, Fredericks WJ, **Schultz DC**, Proctor M, Sekido Y, Minna J, Chernova TA, Wilkinson KD, Avrutskaya AV, Leadon SA. BRCA1-Associated Ubiquitin Hydrolase Required for Transcription-Coupled Repair of Oxidative DNA Damage. (Submitted, 2001).

Schultz DC, Ayyanathan K, Rauscher III FJ. The KRAB-ZFP:KAP1 Repression System Targets Histone H3-K9 Methylation by SETDB1 and HP1 recruitment to Stably Silence Transcription of Endogenous, Euchromatic Promoters. (In preparation)

Schultz DC, Lechner MS, Bochar D, Rauscher III FJ, Shiekhattar R. Biochemical Purification of a HP1a Protein Complex: Implications for DNA Replication Dependent Maintenance of Heterochromatin. (In preparation)

Grants

NIH-1KO1CA94047-01 (Howard Temin Award) "Functions of a novel SET protein in KRAB:KAP1 repression" (**Submitted 2/1/01**)

Abstracts

Schultz DC, Capili AD, Borden KLB, Rauscher III FJ. The KRAB-ZFP:KAP-1 Repression System Recruits the Histone Deacetylase Complex, NuRD, via an Interaction Between the PHD and Bromodomains of KAP1 and the Mi-2 Subunit. (Kestone Symposia-"Mechanisms of Eukaryotic Transcriptional Regulation," Santa Fe, NM, February 2001).

Schultz DC, Ayyanathan K, Rauscher III FJ. SETDB1: A novel SET domain protein that contains histone H3-lysine 9 specific methylase activity, and recruits HP1 to endogenous, euchromatic targets. (Submitted, Kestone Symposia-"Epigenetics in Development and Disease," Taos, NM, February 2002).

Schultz DC, Ayyanathan K, Rauscher III FJ. SETDB1: A new SET domain protein with histone H3, lysine-9-specific methyltransferase activity that contributes to silencing endogenous, euchromatic genes by recruiting HP1. (Submitted, 2002 AACR Annual meeting-San Francisco, CA, April 2002).

Invited Interviews

Iowa State University, Department of Biochemistry and Molecular Biology. Ames, IA. 2/16/01
LSU-Shreveport, Department of Biochemistry. Shreveport, LA. 2/22/01
Tulane University Medical School, Department of Pharmacology. New Orleans, LA. 3/9/01
MCP/Hahnemann, Department of Biochemistry. Philadelphia, PA. 3/15/01
Case Western Reserve University, Department of Pharmacology. Cleveland, OH. 3/19/01
UMDNJ, Department of Biochemistry. Newark, NJ. 3/26/01
University of Michigan, Department of Human Genetics. Ann Arbor, MI. 3/30/01
Florida International University, Department of Biological Sciences. Miami, FL. 5/7/01
Roswell Park Cancer Institute, Department of Cancer Genetics. Buffalo, NY. 5/16/01
St. Jude's Children's Hospital, Department of Biochemistry, Memphis, TN. 5/22/01
Northwestern School of Medicine, Department of Molecular Pharmacology and Biological Chemistry. Chicago, IL. 6/14/01

Conclusions:

The goal of this research fellowship was to further broaden my training in molecular biology/biochemistry with a particular focus on understanding the biochemical functions of novel gene products, which may have a role in the etiology of human disease. The findings of the current research have greatly fostered the development of a new research program, addressing the role of epigenetic regulation of gene expression in breast cancer, which I will pursue as an independent investigator. I have made sixty-five applications for a tenure-track faculty position as an assistant professor. I had

eleven independent interviews from this application process and was formally offered a tenure-track faculty position from 6 different academic institutions. I have formally accepted a position in the Department of Pharmacology at the Case Western Reserve School of Medicine. The **long-range goal** of this lab will be to understand mechanisms epigenetic gene regulation during both normal development and pathogenesis. The immediate **focus** will continue to use both genetic and biochemical approaches to evaluate the role of chromatin modifying machinery in epigenetic gene regulation. The **rationale** for this research is that once the mechanics of transcriptional repression of gene promoters at the chromatin level is known, new and innovative approaches, both genetic and pharmacologic, can be designed to reverse aberrant, gene expression in breast cancers.

References:

1. **Cairns, B. R.** 2001. Emerging roles for chromatin remodeling in cancer biology *Trends Cell Biol.* **11**:S15-21.
2. **Capili, A. D., D. C. Schultz, I. F. Rauscher, and K. L. Borden** 2001. Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains *Embo J.* **20**:165-77.
3. **Friedman, J. R., W. J. Fredericks, D. E. Jensen, D. W. Speicher, X. P. Huang, E. G. Neilson, and F. J. I. Rauscher** 1996. KAP-1, a novel corepressor for the highly conserved KRAB repression domain *Genes and Development.* **10**:2067-2078.
4. **Kirschmann, D. A., R. A. Lininger, L. M. Gardner, E. A. Seftor, V. A. Otero, A. M. Ainsztein, W. C. Earnshaw, L. L. Wallrath, and M. J. Hendrix** 2000. Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer *Cancer Res.* **60**:3359-63.
5. **Margolin, J. F., J. R. Friedman, W. K. Meyer, H. Vissing, H. J. Thiesen, and F. J. I. Rauscher** 1994. Kruppel-associated boxes are potent transcriptional repression domains *Proceedings of the National Academy of Sciences of the United States of America.* **91**:4509-4513.
6. **Peng, H., G. E. Begg, S. L. Harper, J. R. Friedman, D. W. Speicher, and F. J. r. Rauscher** 2000. Biochemical analysis of the KRAB transcriptional repression domain:spectral, kinetic and stoichiometric properties of the KRA-KAP-1 complex *J Biol Chem.* **275**:18000-18010.
7. **Peng, H., G. E. Begg, D. C. Schultz, J. R. Friedman, D. E. Jensen, D. W. Speicher, and F. J. Rauscher, 3rd** 2000. Reconstitution of the KRAB-KAP-1 Repressor Complex: A Model System for Defining the Molecular Anatomy of RING-B Box-coiled-coil Domain- mediated Protein-protein Interactions *J Mol Biol.* **295**:1139-1162.
8. **Rea, S., F. Eisenhaber, D. O'Carroll, B. D. Strahl, Z. W. Sun, M. Schmid, S. Opravil, K. Mechtler, C. P. Ponting, C. D. Allis, and T. Jenuwein** 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases [see comments] *Nature.* **406**:593-9.
9. **Ryan, R. F., D. C. Schultz, K. Ayyanathan, P. B. Singh, J. R. Friedman, W. J. Fredericks, and F. J. Rauscher, 3rd** 1999. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing *Mol Cell Biol.* **19**:4366-78.
10. **Schultz, D. C., J. R. Friedman, and F. J. Rauscher, 3rd** 2001. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD *Genes Dev.* **15**:428-43.
11. **Sorlie, T., C. M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M. B. Eisen, M. van de Rijn, S. S. Jeffrey, T. Thorsen, H. Quist, J. C. Matese, P. O. Brown, D.**

- Botstein, P. Eystein Lonning, and A. L. Borresen-Dale** 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications Proc Natl Acad Sci U S A. **98**:10869-74.
12. **Zheng, L., H. Pan, S. Li, A. Flesken-Nikitin, L. P. Chen, G. T. Boyer, and H. W. Lee** 2000. Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1.[In Process Citation] Mol Cell. **6**:757-68.

Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains

Allan D.Capili, David C.Schultz¹,
Frank J.Rauscher,III^{1,2} and
Katherine L.B.Borden²

Structural Biology Program, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York University, New York, NY 10029 and ¹The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

²Corresponding authors
e-mail: kathy@physbio.mssm.edu or Rauscher@wistar.upenn.edu

A.D.Capili and D.C.Schultz contributed equally to this work

Plant homeodomain (PHD) domains are found in >400 eukaryotic proteins, many of which are transcriptional regulators. Naturally occurring point mutations or deletions of this domain contribute to a variety of human diseases, including ATRX syndrome, myeloid leukemias and autoimmune dysfunction. Here we report the first structural characterization of a PHD domain. Our studies reveal that the PHD domain from KAP-1 corepressor binds zinc in a cross-brace topology between anti-parallel β -strands reminiscent of RING (really interesting new gene) domains. Using a mutational analysis, we define the structural features required for transcriptional repression by KAP-1 and explain naturally occurring, disease-causing mutations in PHD domains of other proteins. From a comparison of this PHD structure with previously reported RING and LIM (Lin11/Is1-1/Mec-3) structures, we infer sequence determinants that allow discrimination among PHD, RING and LIM motifs.
Keywords: KAP-1/LIM/PHD/RING/zinc finger

Introduction

Biological processes depend on the spatial and temporal targeting of specific macromolecular interactions. The presence of highly conserved amino acid sequence motifs and their structural arrangement within a novel protein can provide the first clue to the protein's function. Modular motifs within multi-domain proteins often specify interacting partners and the identification of these interactions has been beneficial in defining biochemical functions of particular proteins and their cellular pathways. Furthermore, these modular domains are often targets of mutations that disrupt the cellular pathways in which they function. Identification of modular domain type through sequence homology studies alone can lead to ambiguous domain-type assignment, making prediction of function a difficult enterprise. Thus, biophysical characterization and determination of the three-dimensional (3D) structure are essential for understanding cellular and biological function.

The plant homeodomain (PHD) or leukemia associated protein (LAP) domain is a relatively small motif of ~60 amino acids that is found in >400 eukaryotic proteins, many of which are believed to be involved in the regulation of gene expression, including the KAP-1/TIF1 β , WCRF/WSTF, Mi-2 and CBP/p300 families (Aasland *et al.*, 1995). It has been suggested that this domain is involved in protein-protein interactions related to a possible role in chromatin-mediated regulation of gene expression (Jacobson and Pillus, 1999). Although no direct role in transcriptional processes has been reported for this domain, recent reports indicate that the extended PHD domain of AF10 is necessary for homo-oligomerization, an event required for the ability of the AT hook motif in AF10 to bind DNA (Linder *et al.*, 2000).

The biological importance of the PHD domain is underscored by its involvement in the pathogenesis of several human disorders. Clinically relevant missense mutations in the PHD domain of the ATRX protein result in α -thalassemia and mental retardation (Gibbons *et al.*, 1997; Rinderle *et al.*, 1999). Germline nonsense mutations in the *AIRE* gene result in truncated proteins where one or both of the PHD domains are deleted in patients with autoimmune polyglandular syndrome type 1 (APECED) (The Finnish-German APECED Consortium, 1997). Somatic acquired point mutations in the PHD domain of ING1 have been identified in head and neck squamous cell carcinomas (Gunduz *et al.*, 2000). Chromosomal rearrangements that delete this motif in proteins such as MLL, CBP, MOZ and AF10 result in myeloid leukemias (Jacobson and Pillus, 1999). Furthermore, genes encoding PHD domain proteins have been identified in the critical deletion regions of several contiguous gene deletion syndromes such as Williams syndrome (WSTF) and the immunodeficiency syndrome ICF (DMNT3B) (Lu *et al.*, 1998; Aapola *et al.*, 2000). The prevalence of disease-causing mutations in PHD domains suggests that they play a basic and essential role in the normal function of human cells.

The PHD domain is a motif characteristically defined by seven cysteines and a histidine that are spatially arranged in a C4HC3 consensus with intervening sequences of varying length and composition (Aasland *et al.*, 1995). This particular arrangement of amino acids is highly homologous to the RING (really interesting new gene) and LIM (Lin11/Is1-1/Mec-3) domains (Figure 1A) (Borden and Freemont, 1996; Dawid *et al.*, 1998). In both the RING and the LIM domain, these conserved cysteine and histidine residues are utilized to bind two zinc atoms, a process cooperatively coupled to the folding of the domains. The LIM uses a sequential zinc ligation scheme, where the first and second pair of metal ligands create the first zinc-binding site, while the third and fourth pair form the second site (Figure 1B). In contrast, the RING domain

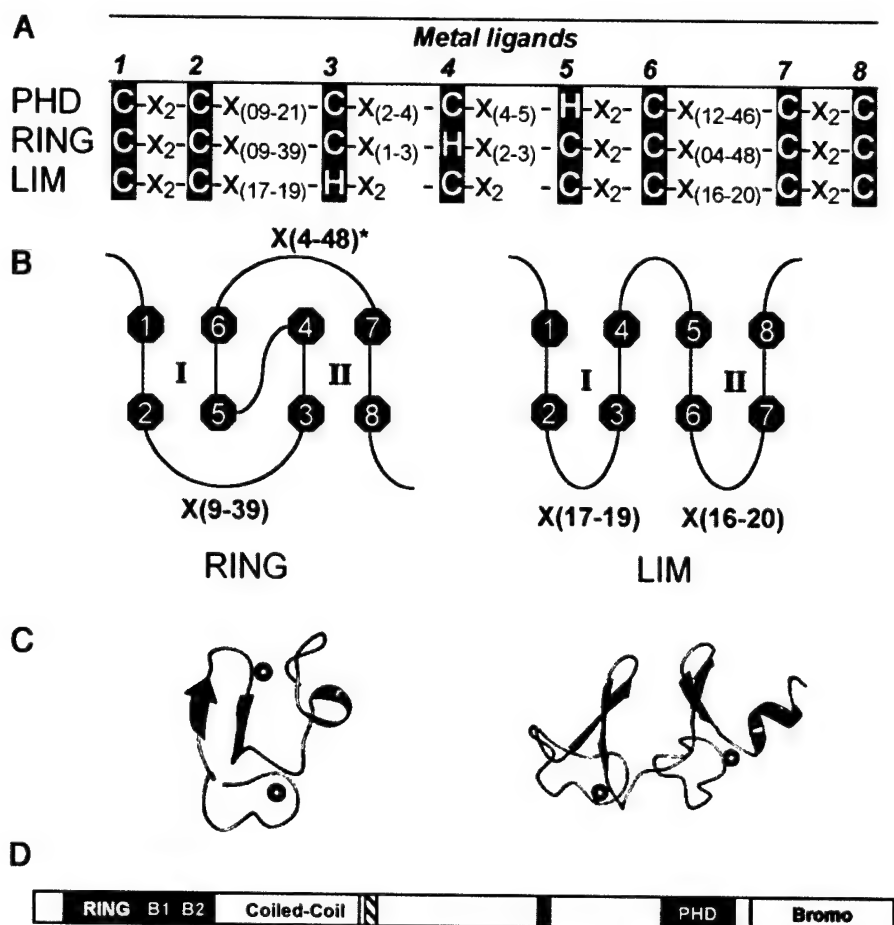


Fig. 1. (A) The consensus sequences that define the PHD, RING and LIM domains. C, cysteine; H, histidine; X, any residue. Above the consensus sequence the number of each metal ligand, *ml*, is given. The first and second pairs of sequential metal ligands are in dark blue, while the third and fourth pairs are in magenta. (B) Demonstration of zinc ligation patterns found in RING and LIM domains. The RING uses a cross-brace ligation scheme while the LIM uses a sequential ligation scheme. Numbers correlate to the number of the metal ligand as defined in (A). Zinc atoms are represented by gray ovals and the zinc-binding sites are denoted by roman numerals. (C) Ribbon diagrams of the three-dimensional structures. Zinc atoms are represented by spheres. Structures are from the RING of PML (1BOR) and for LIM (1A7I). (D) A schematic representation of the domains in the KAP-1 protein. RING, the RING domain; B1 and B2, two adjacent B-boxes; Coiled-Coil, a leucine coiled-coil domain; hatched box, the TIFF signature sequence; black box, the HP1-binding site; PHD, the PHD domain; Bromo, the bromodomain.

utilizes a unique cross-brace ligation topology, where the first and third pair of metal ligands form the first zinc-binding site (site I), while the second and fourth pair form the second site (site II) (Figure 1B). The different arrangements of metal ligands result in dramatically different 3D structures (Figure 1C). The choice of ligation topology is undoubtedly critical for proper folding and domain functionality. Given the sequence similarity amongst PHD, RING and LIM domains, one is unable to predict the ligation scheme and fold by sequence analysis alone.

Here, we report the first solution structure of the PHD domain from the KAP-1 corepressor (also known as TIF1 β or KRIP1), a universal corepressor for the KRAB-zinc finger superfamily of transcriptional repressors (Friedman *et al.*, 1996). An N-terminal RBCC domain (RING domain, B-boxes and coiled-coil region) is both necessary and sufficient for interaction of KAP-1 with the KRAB repression module (Peng *et al.*, 2000a,b). Amino acid sequences C-terminal to the RBCC domain represent at least two independent repression domains. A direct

interaction between KAP-1 and the HP1 family of proteins suggests that KRAB-zinc finger proteins repress transcription through heterochromatin-mediated gene silencing (Ryan *et al.*, 1999; Lechner *et al.*, 2000). The PHD domain is found on the C-terminus of KAP-1, adjacent to a bromodomain defining an independent repressional unit (Figure 1D). These two domains are found together in ~30 PHD-containing proteins, suggesting that they may functionally interact (Le Douarin *et al.*, 1995, 1996; Friedman *et al.*, 1996; Venturini *et al.*, 1999; Bochar *et al.*, 2000; Jones *et al.*, 2000). We demonstrate that the PHD forms an autonomously folding domain that binds two zinc atoms in a cross-brace RING-like arrangement. Not only are the zinc ligation schemes identical, but also the tertiary topologies of the PHD and RING domains are remarkably similar. From the comparison of PHD, RING and LIM domains, it is possible to derive unique structural determinants. Structure-based site-directed mutational analysis reveals structural features of the PHD domain that are critical for the ability of KAP-1 to repress transcription. Furthermore, we are able to rationalize the structural

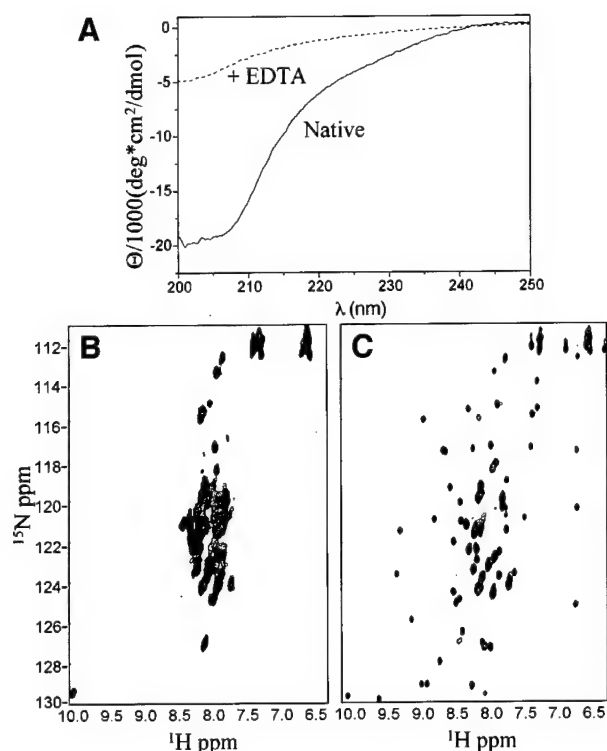


Fig. 2. (A) CD spectrum of untagged KAP-1 PHD prior to addition of EDTA (solid line) and after addition (dashed line). (B and C) $^1\text{H}/^{15}\text{N}$ -HSQC of KAP-1 PHD produced in minimal media not supplemented (B) or supplemented with zinc (C).

consequences of mutations in the PHD domains of other proteins such as ATRX and ING1, where these mutations correlate with human disease.

Results and discussion

The PHD domain requires zinc for folding

The conservation of eight potential metal ligands in the PHD family suggested that it is a zinc-binding protein. In order to analyze the chemical and physical characteristics of the PHD domain, we expressed the KAP-1 PHD (amino acids 618–679) in *Escherichia coli* and purified the protein to homogeneity using affinity and size exclusion chromatographies. Analytical ultracentrifugation experiments suggest that >90% of the PHD protein in solution is a monomer (data not shown). To determine the identity and stoichiometry of metal bound by the PHD domain, we used inductively coupled plasma (ICP) spectrometry. These measurements indicate that a 3.0 mM solution of KAP-1 PHD protein contains 5.2 mM ($\pm 3.3\%$) zinc. This ratio of one protein molecule to 1.7 zinc atoms indicates that the PHD binds two zinc atoms per protein molecule, potentially utilizing its eight conserved cysteine and histidine residues for metal ligation.

Based on this result, we investigated the role of zinc in structure formation of the PHD domain using circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopies. The CD spectrum of KAP-1 PHD denotes a folded protein with substantial β -sheet and no significant helical content (Figure 2A). Introduction of the metal chelating agent EDTA results in complete loss of second-

ary structure. Addition of ZnCl_2 , in excess of EDTA, results in re-folding of the domain (data not shown). Similarly, $^1\text{H}/^{15}\text{N}$ -HSQC (heteronuclear single quantum correlation) NMR experiments indicate that the KAP-1 PHD domain requires zinc for proper folding (Figure 2B and C). In these experiments, the PHD domain was expressed in minimal media in the presence or absence of zinc and purified identically. The spectrum of the sample expressed in the absence of zinc shows poor dispersion of amide proton chemical shifts, indicative of an unfolded protein (Figure 2B). In contrast, the sample expressed in the presence of zinc produces a spectrum revealing a substantial increase in chemical shift dispersion, a decrease in line widths, and the appearance of virtually all expected backbone amide resonances; all hallmarks of a well-folded protein (Figure 2C). Thus, like RING and LIM domains, the binding of two zinc atoms is necessary for folding of the PHD domain.

The structure of the KAP-1 PHD domain reveals a cross-brace zinc ligation scheme

We determined the solution structure of the KAP-1 PHD domain using standard homonuclear and heteronuclear NMR techniques. Initial NMR studies were conducted on an N-terminal His-tagged construct of KAP-1 (amino acids 618–679) containing the entire PHD domain. A comparison of 2D $^1\text{H}/^{15}\text{N}$ HSQC and NOESY (nuclear Overhauser effect spectroscopy) spectra of tagged and untagged proteins revealed that the two spectra were identical apart from resonances corresponding to the tag (data not shown). Thus, the presence of this N-terminal His tag had no influence on the structure of the PHD domain.

An initial set of structures was generated without reference to metal ligation in order to determine the residues involved in zinc binding. These structures indicated a cross-brace ligation scheme with residues C628, C631, H648 and C651 forming one zinc-binding site (site I) and C640, C643, C666 and C669 forming the other (site II). These sites were partially defined by the individual NOEs observed between residues implicated in zinc ligation. For instance, long-range NOEs were observed between C666 and C640, C631 and C651, and C640 and C669; in addition, several NOEs between residues adjacent to the zinc ligands were observed. Subsequent structure calculations included Zn atoms and additional constraints defining tetrahedral coordination. To ensure that a cross-brace was the correct zinc ligation scheme, alternative schemes were calculated but none satisfied the experimental constraints. The final ensemble is displayed in Figure 3A and all atoms are depicted in Figure 3B. Statistics of the ensemble are given in Table I.

The structure of the KAP-1 PHD domain reveals a globular domain binding two Zn atoms in a distinguishing cross-brace fashion (Figure 3). In this arrangement, residues C628, C631, H648 and C651 form a single zinc-binding site (site I), while C640, C643, C666 and C669 form the second site (site II) (refer to Figure 3E). The formation of site I begins with an eight residue loop (residues 627–634), which continues into an extended region (residues 635–636) followed by the first β -strand (β_1 ; residues 637–640). Site II is then created by a turn (residues 641–644) that ends in the second β -strand (β_2 ; residues 645–647) running anti-parallel to β_1 . The first

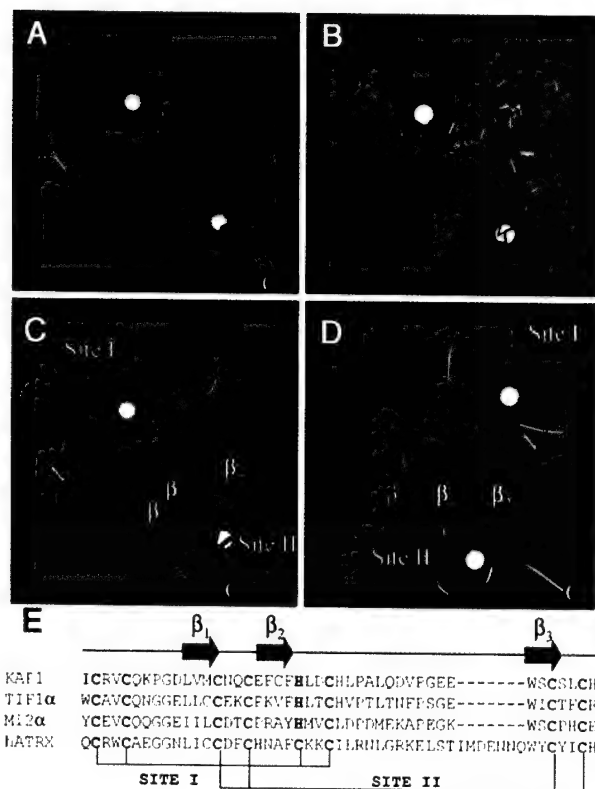


Fig. 3. The KAP-1 PHD structure. (A) α -carbon overlay of 10 KAP-1 PHD domain structures. The residues N-terminal to I627 and C-terminal to H670 are disordered and not shown. The average position of the two zinc atoms is represented by white spheres. The blue portion of the ensemble represents the flexible hinge region described in the text. Structural statistics are given in Table I. (B) All-atom view of KAP-1 PHD in the same orientation as in (A). The main chain is colored in gray. Side chains are colored as follows: hydrophobic in yellow, polar (non-charged) in cyan, polar (acidic) in red, and polar (basic) in blue. (C and D) Ribbon diagram of the KAP-1 PHD domain. The β -strands are shown as arrows and zinc ligands in green. (E) Sequence alignment of PHD domains and a schematic depicting zinc ligation.

zinc-binding site is completed by another loop (residues 648–652). This portion is followed by an extended flexible region (residues 653–663; shown in blue, Figure 3A) demarcated by two proline residues (P654 and P660), which appear to act as a hinge relative to the rest of the domain (Figure 3A). This flexible region is followed by a third β -strand (β_3 ; residues 664–666), leading to the completion of the second zinc-binding site (residues 666–670). Numerous hydrophobic interactions appear to stabilize this structure. F647 is at the center of the hydrophobic core packing against W664, V638, L656 and H652. Interestingly, the proton resonances for the aromatic ring of F647 are not degenerate, indicating that the phenyl group is not free to rotate in the core, suggesting that the core is tightly packed. Analysis of the electrostatic surface potential reveals two areas of discrete charge (Nicholls *et al.*, 1991). Most notably, the flexible hinge (residues 653–663) is lined with several Glu and Asp residues, contributing to a negatively charged surface, and the presence of Arg629 and Lys633 results in a positively charged cluster around site I. Ongoing mutational analyses

Table I. Structural statistics

Restrains for structure calculation (residues 627–670)	
total restraints	385
total NOE restraints	317
intra-residue	78
sequential	118
medium range ($2 < i-j < 4$)	26
long-range ($ i-j \geq 5$)	95
dihedral angle (ϕ)	20
H-bond restraints	12
zinc distance restraints	20
zinc angle restraints	16
Final energies (kcal/mol)	
E_{total}	37.1 ± 1.1
E_{NOE}	0.30 ± 0.21
E_{cdh}	0.05 ± 0.04
Coordinate precision (627–653, 664–670)	
r.m.s.d. of backbone atoms	$0.73 \pm 0.16 \text{ \AA}$
r.m.s.d. of all heavy atoms	$1.48 \pm 0.18 \text{ \AA}$
Procheck analysis (627–670)	
most favored regions	36.0%
additionally allowed	45.9%
generously allowed	14.9%
disallowed region	2.4%

will be utilized to determine the importance of these features to KAP-1 PHD function. In terms of charge and topology, no structural features typical of nucleic acid binding proteins are observed, such as those present in the Kruppel-like or GATA-like zinc fingers (Klevit, 1991; Omichinski *et al.*, 1993). This suggests that the PHD is not a nucleic acid binding domain.

The PHD domain structurally resembles a RING

Unlike the sequential zinc ligation pattern used by the LIM domains, the KAP-1 PHD domain structure shows a zinc ligation pattern similar to RING domains. Therefore, we carried out a detailed comparison between the PHD domain and three previously reported RING domain structures, from the promyelocytic leukemia protein PML, the immediate early equine herpes virus protein IEEHV (also known as ICP0) and the recombination protein RAG1 (Protein Data Bank codes 1BOR, 1CHC and 1RMD, respectively; Barlow *et al.*, 1994; Borden *et al.*, 1995a; Bellon *et al.*, 1997). These proteins have no sequence homology outside of conserved metal-binding residues. Strikingly, in all cases the inter-zinc distance is $\sim 14 \text{ \AA}$, presumably because the central β -strand of these molecules is the same length (β_2 in KAP-1 PHD) and all use the cross-brace ligation scheme. Because PHD and RING domains use different permutations of cysteines and histidines for metal ligation, for clarity we denote the i th conserved metal-ligating residue along the primary sequence by ml_i . Notably, a conserved hydrophobic core residue (F647) is located in β_2 , just N-terminal to ml_5 . Inspection of these structures indicates that the first zinc-binding site and anti-parallel β -strands ($\beta_1\beta_2$) of PHD overlay strikingly well with the three RING structures (Figure 4). An α -carbon backbone alignment of RING and KAP-1 PHD structures for site I overlay almost exactly, as do the zinc atoms. The root mean square difference (r.m.s.d.) of the α -carbon backbone for residues around site I is 1.4 \AA when compared with PML, and 1.3 \AA when compared with IEEHV or RAG1 (Figure 4C). PML,

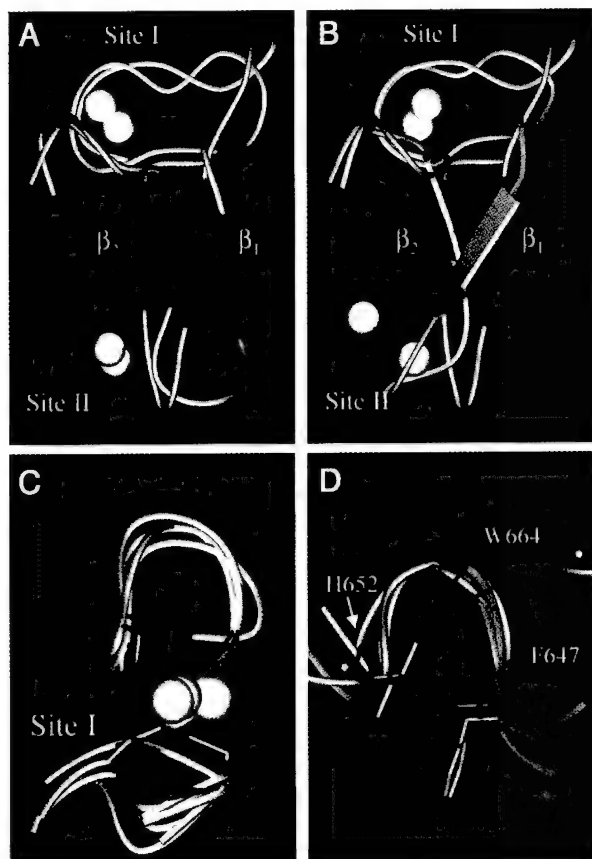


Fig. 4. Comparison of the KAP-1 PHD, PML RING, IEEHV RING and RAG1 RING. Superposition of KAP-1 PHD (with blue β -strands, aa 627–652) and PML RING (magenta β -strands, aa 56–81) (A) and RAG1 RING (yellow β -strands, aa 292–317) (B) from the first metal ligand to the sixth metal ligand. The white spheres represent zinc atoms with the upper zinc atom being site I. The orientation of KAP-1 PHD is the same in both panels. (C) Superposition of zinc-binding site I for KAP-1 (627–632, 647–652), PML (56–71, 76–81), IEEHV (7–12, 28–33) and RAG1 (292–297, 312–317). The metal ligands are colored according to protein: KAP-1 in blue, PML in magenta, IEEHV in green and RAG1 in yellow. (D) Superposition of the conserved hydrophobic core residues N-terminal to metal ligand 5 and C-terminal to metal ligand 6 (L76 and L81 in PML, F28 and I33 in IEEHV, and F312, I317, F647 and H652 in PHD). The side-chains are colored as in (C). The core residues from PHD are noted for clarity. The conserved tryptophan within the PHD family (W664 in KAP-1) is seen here inserting between the other core residues, repositioning the core.

IEEHV and RAG1 RINGs have a large insertion along the β_1 strand relative to KAP-1 PHD (Figure 5). Comparison of residues just N-terminal to ml_1 through to just C-terminal to ml_6 , which include residues in the hydrophobic core, results in r.m.s.d. values between KAP-1 and PML of 2.0 Å, between KAP-1 and IEEHV of 2.1 Å, and between KAP-1 and RAG1 of 1.9 Å. The sequence identity in this region is 20% between PML and KAP-1, 22% between IEEHV and KAP-1, and 24% between RAG1 and KAP-1 (Figure 4A and B). Normally, r.m.s.d. values of ~2 Å are expected for proteins with sequence identities >30% (Flores *et al.*, 1993). Thus, these levels of similarity are striking considering there is virtually no sequence conservation between these domains outside of their conserved zinc ligands.

Like the PHD family, the RING family shows substantial structural plasticity between ligands ml_6 and ml_7 , where the chain completes site I and continues towards site II. In PHD domains, the spacing varies from 12 to 46 residues, and in RING domains, the spacing varies from 4 to 48 residues. The RINGs of IEEHV, RAG1 and BRCA1 have large inserts in this region, forming a helix. However, RINGs with shorter inserts, e.g. PML and BARD1, do not have a helix in this region (information for BRCA1 and BARD1; P. Brozovic and R. Klevit, personal communication; reviewed in Borden, 1998). In contrast, this region in the KAP-1 PHD contains the flexible hinge (residues 653–663). Although this insert is comparable in size to that of RINGs containing a helix, the propensity for this region to adopt a helical conformation is highly unlikely due to the presence of two proline residues (P654 and P660).

A major structural difference between PHD and RING domains is found in the hydrophobic core. Residues that form the hydrophobic core of the RING structures are N-terminal to ml_5 and C-terminal to ml_6 (L76 and L81 in PML, F28 and I33 in IEEHV, F312 and I317 in RAG1; see also Figures 4D and 5) (Barlow *et al.*, 1994; Borden *et al.*, 1995a; Borden, 1998). Conserved hydrophobic residues at the same positions within the amino acid sequence are also found in the PHD (F647, N-terminal to ml_5 ; and H652, C-terminal to ml_6). However, there exists an additional conserved hydrophobic residue in PHD: a tryptophan two residues N-terminal to ml_7 (W664 in KAP-1; Figures 4D and 5). This tryptophan, which is highly conserved throughout the PHD family, inserts between the core residues N-terminal to ml_5 and C-terminal to ml_6 and thereby forms part of the hydrophobic core. In RINGs, no hydrophobic core residue exists at the position two residues before ml_7 (Figures 4D and 5). Rather, in all RING structures reported, the amino acid at this position is actually solvent exposed (Borden, 1998). This additional core residue appears specific to the PHD family. The presence of W664 alters the arrangement of the core where H652, which is C-terminal to ml_6 , is not as central to the core as this residue is in the RING domain. Additionally, the trace of the main chain is virtually unaltered relative to the RING fold (Figure 4D). Ligands ml_3 and ml_4 in site II are positioned almost identically in both PHDs and RINGs. In summary, both RING and PHD domains have conserved hydrophobic residues that are in the same sequence position and are found in the core of the domains. However, PHD accommodates an additional core residue, W664.

Aside from differences in the hydrophobic core, there are significant differences in the main-chain trace between ml_6 and ml_7 in PHD compared with RING domains. At H652, just C-terminal to ml_6 , the main-chain trace deviates significantly from RING domains. This difference appears to result from the participation of W664 (two residues N-terminal to ml_7) in the hydrophobic core. This alters the position of ml_7 and ml_8 relative to the RINGs. These differences suggest that the PHD domain, although structurally similar to the RING in part, could bind structurally distinct partners and display unique biological

based sequence determinants that would enable one to predict whether a given amino acid sequence is PHD-, RING- or LIM-like in structure. The major structural determinant appears to be based on the zinc ligation scheme utilized. Sequence alignments for selected members of the PHD, RING and LIM families are given in Figure 5. For RING and LIM domains, conserved hydrophobic core residues determined through inspection of the structures are shown. In these cases, residues that form the hydrophobic core are shown in yellow, site I in magenta and site II in blue. These alignments suggest that proteins with conserved hydrophobic residues at positions N-terminal to ml_5 and C-terminal to ml_6 utilize a cross-brace ligation scheme as in the PHD/RING structures, whereas proteins with hydrophobic residues N-terminal to ml_3 and C-terminal to ml_4 utilize a sequential LIM-like ligation scheme. Alignments indicate that in the PHD/RING cross-brace schemes, the presence of a tryptophan residue two residues N-terminal to ml_7 may be diagnostic of a PHD fold. In contrast, LIMs have additional conserved hydrophobic core residues that are not found in PHD or RING (Figure 5). Furthermore, residue spacing in the LIM domain is much more highly conserved than in PHDs or RINGs (Figure 1A).

Analysis of this structure allows one to distinguish between conserved hydrophobic residues observed by sequence information and conserved hydrophobic residues that additionally form the core of the domain. Some hydrophobic residues conserved within the PHD family do not participate in formation of the hydrophobic core. Residues positioned one and two amino acids N-terminal to ml_3 , Leu 637 and Val 638, are conserved as hydrophobic residues in the family (Figure 5) but are not part of the core. Mutation of Leu637 to Ala does not disrupt the transcriptional repression activity of KAP-1 to the same extent as mutation of conserved hydrophobic core residues, which results in almost complete loss of transcriptional activity and an unfolded protein (Figures 6 and 7; Table II).

For RING and LIM domains, flexibility in ligating residue type has been observed, e.g. His can replace Cys and even Asp can be used as a ligand. We examined the importance of specific types of zinc ligands to the fold of the PHD domain by examining the ability to repress transcription. Initially, we swapped the type of zinc ligand, e.g. His for Cys, at ligand position ml_5 (648), and determined the ability of KAP-1 to act as a transcriptional repressor (Figure 6B; Table II). This mutation has little effect, indicating that the type of ligating residue, at least at this position, is not crucial. However, substitution of Ala for His at ml_5 completely destroys the transcriptional repression properties of the KAP-1 PHD domain (Figure 6B; Table II). Intriguingly, in studies where

KAP-1 was modified (Cys643His648 to His643Cys648) so that it used a RING-like zinc ligation scheme (Figure 1), there was a substantial loss of transcriptional activity relative to wild-type protein (Figure 6). Furthermore, mutation of Cys643 to His results in reduction in this activity but not to the same extent as the double mutation. Thus, ligand type may be critical at some positions within the sequence, whereas the choice of ligand type appears more flexible at other positions.

Structural integrity of the PHD domain is essential for KAP-1 to repress transcription

In order to define structural features of the PHD domain important for the transcriptional repression by KAP-1, mutational analysis was carried out on a GAL4-KAP-1 (619–835) expression construct (Figure 6A). This construct encodes both the PHD domain and the bromodomain of KAP-1, which together comprise an independent repression domain where both domains are required for this biological activity (Figure 6B and C; Table II; D.C.Schultz, J.R.Friedman and F.J.Rauscher, III, submitted). The positioning of the PHD domain adjacent to a bromodomain has been observed in several proteins; however, most PHD-containing proteins do not contain a bromodomain, suggesting that the PHD is an independent structural unit. Yet, in KAP-1, it is possible that the bromodomain could alter the fold of the PHD domain and thereby modulate its biological activity. In order to address whether the bromodomain could alter the structure of the PHD, we carried out additional NMR studies on a KAP-1 PHD–bromodomain construct. In these experiments, the chemical shifts of the resonances corresponding to the PHD domain appear largely identical to resonances in constructs containing only the PHD domain, indicating that the bromodomain does not substantially alter the fold of the PHD domain (our unpublished observations). Although the bromodomain does not appear to alter the fold of the PHD domain significantly in KAP-1, it may make crucial contacts that modulate its function or cause small-scale structural rearrangements, which may be critical for function.

We monitored the effects of mutations in the PHD domain on transcriptional activity (Figure 6; Table II). Mutations of zinc ligands in either site I (CC628, 631AA or H648A) or site II (C643A and C666A) abolished transcriptional repression, while mutation of the non-zinc-ligating cysteine (C646V) shows no effect. Furthermore, mutations that disrupt the hydrophobic core of the molecule are as debilitating as mutations that disrupt zinc ligation. Consistently, conservative non-disruptive mutations, such as F647Y, in the core do not alter transcriptional repression by KAP-1, whereas F647A abolishes this activity.

Fig. 5. Amino acid sequence alignment of the PHD, RING and LIM families. Site I metal ligands are colored in magenta and site II metal ligands in blue. Conserved hydrophobic core residues are colored in yellow. DDBJ/EMBL/GenBank accession Nos and PDB codes: KAP-1 (U78773), TIF1 α (AAD17258), Mi2 α (Q14839), ATRX (P46100), DNMT3A (BAA95556), ING1 (AAG02578), YN17_YEAST (P50947), RBB2_HUMAN (P29375), YM42_YEAST (Q03214), YMW5_YEAST (Q04779), YA27_SCHPO (Q09698), YAC5_SCHPO (Q09819), AIRE_HUMAN (O43918), CHD4_HUMAN (Q14839), X169_HUMAN (CAA89909), HT31_ARATH (Q04996), PRH_ARATH (P48785), CHD3_CAEEL (Q22516), CHD3_HUMAN (Q12873), YJ89_YEAST (P47156), FALZ_HUMAN (Q12830), YANC_SCHPO (Q10077), YGN1_YEAST (P53127), YAJ8_SCHPO (Q09908), AF17_HUMAN (Q09908), YGN1_YEAST (P53127), HRX_HUMAN (Q03164), PML (1bor), IEEHV (1chc), RAG1 (1RMD), BRCA1 (A58881), BARD1 (NP_00456), c-Cbl (P22681), MDM2 (CAA41684), MDMX (O15151), MAT (S60157), Z (P18541), Mel-18 (P35227), Crp1LIM1 and LIM2 (1B8T), CRIPrat (1JML), Lin-11 (CAA38240), ISL-1 (CAA3749), MEC-3 (S28390), LMX-1 (B46233).

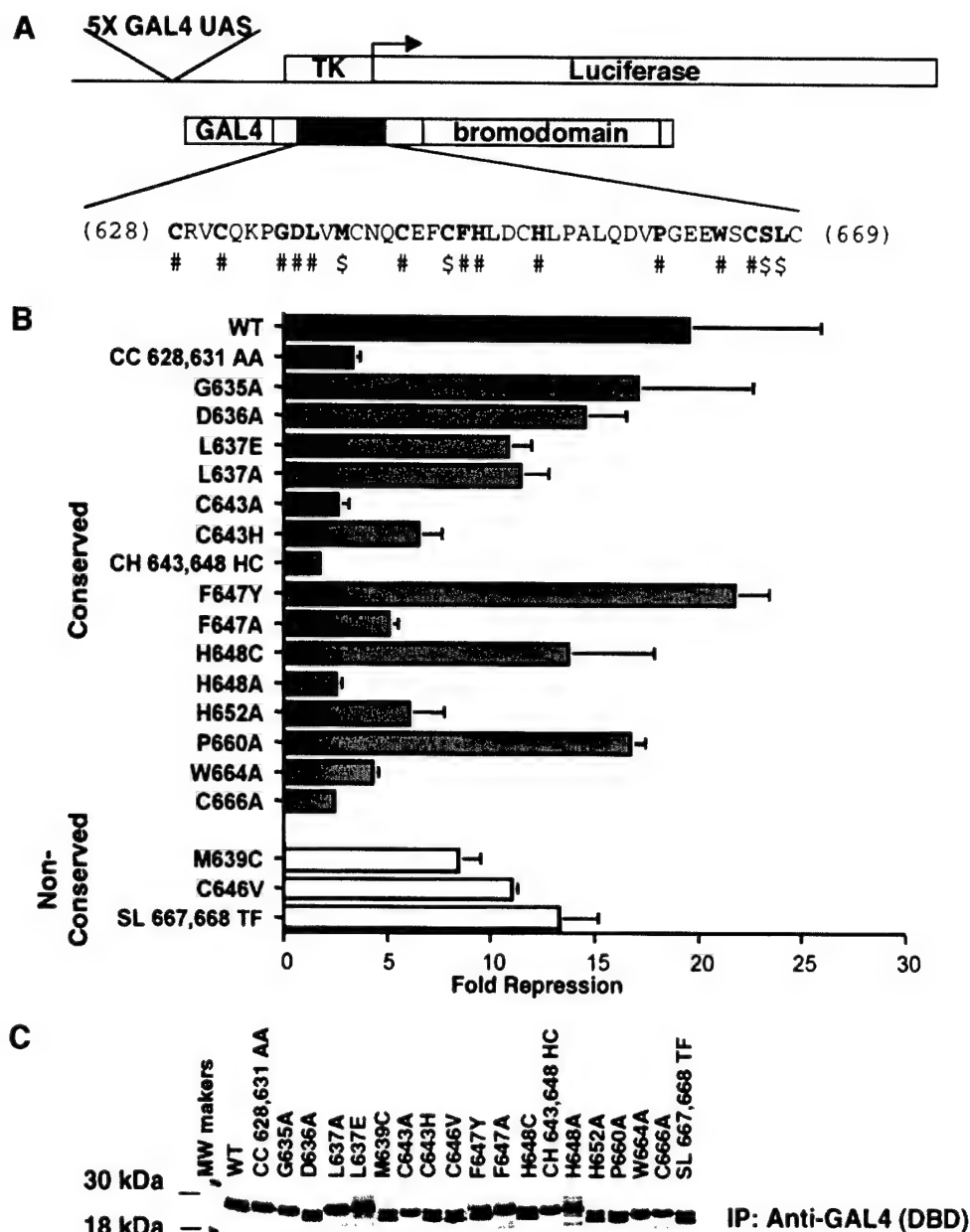


Fig. 6. Mutations in the PHD domain of KAP-1 significantly impair the intrinsic repression activity of KAP-1. (A) Schematic diagram illustrating the reporter plasmid (5X-GAL4-UAS-TK-luciferase) and effector plasmid (GAL4-KAP-1₆₁₉₋₈₃₅). The primary amino acid sequence of the minimal KAP-1 PHD domain is illustrated. #, conserved amino acids that were mutated. \$, non-conserved amino acids in KAP-1 that were mutated to match the corresponding amino acids in TIF1 α /TIF1 γ . Each mutation was made in the context of a GAL4-KAP-1 (619-835) expression construct. (B) Mutations in the KAP-1 PHD domain disrupt its role in transcriptional repression. All experiments were performed in NIH 3T3 cells with 5 μ g of the indicated GAL4 fusion protein and 1 μ g of a 5X-GAL4(UAS)-TK-luciferase reporter. A black bar represents wild type, gray bars represent mutations of conserved amino acid residues, and white bars represent substitutions at non-conserved amino acids. (C) Stable expression of each GAL4 (DBD) antisera (1 μ g).

Mutation of a conserved hydrophobic non-core residue, L637A, does not abolish transcriptional activity of the domain. A mutation that potentially introduces an extra zinc ligand (M639C or P654C) results in a lowered activity, presumably because this 'new' ligand may disrupt the formation of the cross-brace by competition for zinc during the folding process. In the wild-type structure, the M639 side chain is solvent exposed and P654 is part of the flexible hinge region. Reduction in transcriptional repression activity in the case of P654C

or M639C could be due either to misfolding or to a disruption of PHD-bromodomain intermolecular interactions.

To determine the effects that some of these mutations impose on the structural properties of the domain, we expressed the PHD protein containing mutations of three different types. Based on the structure, W664A is expected to disrupt the hydrophobic core, C651F is expected to disrupt metal binding and P654C is expected to cause misfolding by disrupting metal binding through addition of

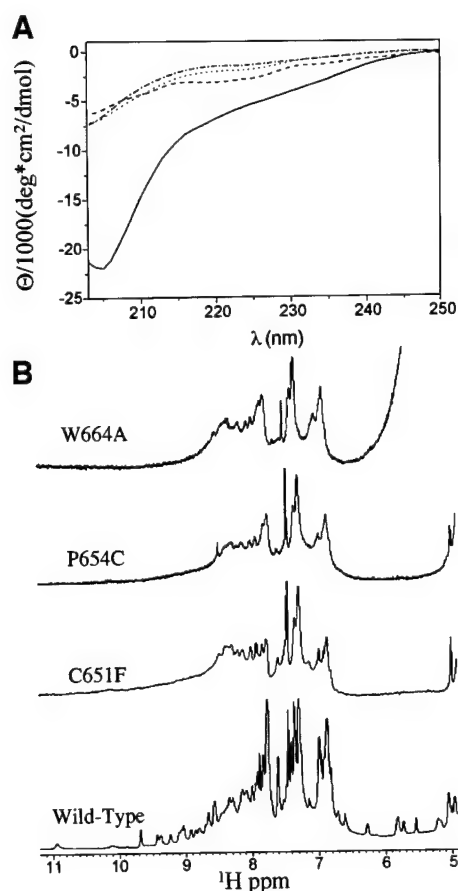


Fig. 7. Some mutations in the PHD domain of KAP-1 disrupt proper folding. (A) Comparison of CD spectra of wild-type KAP-1 PHD (solid line), W664A mutant (dashed/dotted line), P664C mutant (dotted line) and C651F mutant (dashed line) taken at pH 7.5, 25°C. (B) 1D ^1H NMR spectra of wild-type and mutant KAP-1 PHD taken at pH 7.5, 30°C. The spectral regions containing the amide and aromatic proton resonances are shown.

an extra zinc ligand. Each protein was expressed and purified as previously described. As an initial evaluation of the biophysical consequences of these mutations on the structure of the PHD domain, we estimated the hydrodynamic size of each protein by gel filtration. Greater than 90% of the C651F mutant protein eluted from the gel filtration column immediately following the void volume, suggesting that the protein is likely to exist as a soluble aggregate (data not shown), consistent with being largely unfolded. A protein containing the P654C mutation eluted from the column in two distinct peaks. Approximately 50% of the protein eluted from the column just after the void volume, as observed for the C651F mutant protein. The remainder of the protein eluted with a Stokes radius consistent with the monomeric size observed with the wild-type protein (data not shown). The W664A mutant protein eluted from the column as a distribution of species, with a small but significant fraction of the protein eluting at a volume that overlaps with the monomeric size observed with the wild-type protein (data not shown). Fractions corresponding to the monomer for each mutant were collected and further analyzed to determine whether the proteins were folded. NMR and CD studies on the

W664A, C651F and P654C proteins indicated that, as expected, disruption of the hydrophobic core or of zinc binding resulted in unfolded proteins (Figure 7). Analysis of CD spectra of each mutant indicates a substantial loss in molar ellipticity relative to wild type, indicating that the mutants are unfolded (Figure 7A). ^1H NMR studies indicate that there is poor dispersion of the resonances in the amide and aromatic region of the spectra of all three mutants, indicative of an unfolded protein (Figure 7B). Furthermore, there are few NOEs observed in 2D ^1H NOESY experiments, again demonstrating that these proteins are largely unstructured (data not shown). Thus, the ability of the KAP-1 PHD domain to bind zinc and maintain the hydrophobic core is required for its proper folding and ability to repress transcription.

The potential structural consequences of PHD domain mutations in other proteins

Naturally occurring mutations and deletions in the PHD domains of several proteins exemplify the biological importance of this domain. Germline missense mutations in the PHD domain of the ATRX protein predispose individuals to α -thalassemia and mental retardation (Gibbons *et al.*, 1997). Somatic missense mutations found in the PHD domain of ING1 are detected in several types of carcinoma (Gunduz *et al.*, 2000). Analysis of these mutations in light of the KAP-1 PHD structure reveals the basis for their devastating nature (Table II). In the ING1 PHD domain, two mutations of site I are found in squamous cell carcinomas (Gunduz *et al.*, 2000). C631S (where these amino acids are numbered in the context of the KAP-1 PHD), like previous zinc ligand mutations, should destroy zinc binding and structure formation. N632S, adjacent to *ml*₂, may indirectly interfere with zinc binding and, therefore, formation of site I. Two mutations in ATRX (C628R and C651F) disrupt the ability of this domain to ligate zinc. These mutations are expected to result in an unfolded protein, and equivalent mutations in the KAP-1 PHD domain result in a loss of repression (Table II) and unfolding (Figure 7). Another mutation in ATRX introduces an additional potential metal ligand (P654C), which disrupts biological activity through misfolding (Figure 7). Some surface mutations (V630S and Q657E) have no effect on transcriptional activity in these assays; however, these mutations are found in ATRX patients. Thus, these residues may be required by the ATRX protein for mediating specific protein-protein interactions. An interesting feature of the wild-type ATRX PHD domain is that it is a PHD domain with eight cysteines (His at *ml*₅ is replaced by Cys). Mutation of this ligand in KAP-1 (H648C) resulted in only a slight loss of transcriptional activity (Figure 6B), suggesting that either a histidine or a cysteine can be tolerated as a metal ligand at this position. It is interesting to note that ATRX and ING1 proteins do not contain bromodomains, indicating that their disease potential does not arise because of disruption of a PHD-bromodomain interaction. Overall, the functional significance of any of these mutations will depend on context-specific biochemical functions of the given domain. None the less, this panel of mutations will

Table II. PHD mutations and structural consequences

Mutations	Location in KAP-1 PHD structure	Transcriptional repression ^a (%)	Structural consequences
CC628,631AA	Zn ²⁺ ligands of site I	17	loss of metal binding, incorrect folding
G635A	surface, extended region before β_1	87	no obvious structural effect
D636A	surface, extended region before β_1	74	change in surface charge
L637A	surface, conserved hydrophobic	58	no obvious structural effect
L637E	surface, conserved hydrophobic	55	no obvious structural effect
M639C	β_1 , adjacent to metal ligand of site II (C640)	43	extra zinc ligand, may disrupt site II formation
C643A	Zn ²⁺ ligands of site II	13	loss of metal binding, incorrect folding
C646V	β_2 , surface exposed	56	not a zinc ligand, no obvious structural effect
F647Y	core residue, β_2	100	conservative substitution
F647A	core residue, β_2	26	disrupts hydrophobic core
H648C	Zn ²⁺ ligand of site I	70	conservative substitution
H648A	Zn ²⁺ ligand of site I	13	loss of metal binding, incorrect folding
H652A	core residue	31	disrupts hydrophobic core
P660A	flexible hinge	86	no obvious structural effect
W664A	core residue, β_3	22	disrupts hydrophobic core
C666A	Zn ²⁺ ligand of site II	12	loss of metal binding, incorrect folding
SL 667,668 TF	surface, site II	68	no obvious structural effect
ATRX ^{b,c}			
C628R	Zn ²⁺ ligand of site I	15	loss of metal binding, incorrect folding
V630S	surface, site I	85	may be important for protein-protein interactions
C651F	Zn ²⁺ ligand of site I	12	loss of metal binding, incorrect folding
P654C	flexible hinge	42	extra zinc ligand causing incorrect folding
Q657E	surface, flexible hinge	63	change in surface charge
ING1 ^{b,d}			
C631S	Zn ²⁺ ligand of site I	—	loss of metal binding, incorrect folding
N632S	surface, adjacent to metal ligand	—	may disrupt site I formation

^aPercentage compared with transcriptional repression by wild-type KAP-1 PHD.

^bNumbers refer to the residue numbering for KAP-1.

^cResults for ATRX mutations appear elsewhere (D.C.Schultz, J.R.Friedman and F.J.Rauscher, III, submitted).

^dTranscriptional assays were not conducted for ING1 mutations.

serve as a useful tool in future studies in defining new macromolecular interactions and biological functions for the PHD domain.

The PHD domain is likely to be a protein-protein interaction domain

KAP-1 PHD participates in protein-protein interactions with Mi-2 α (D.C.Schultz, J.R.Friedman and F.J.Rauscher, III, submitted), a component of the NuRD histone deacetylase complex (Wade *et al.*, 1998; Brehm *et al.*, 1999). Therefore, KAP-1 PHD may be important for proper spatial and temporal scaffolding of the repressor complex. This association is likely to be critical for transcriptional repression by KAP-1. Consistent with our results, recent work indicates that the AIRE PHD domain is required for formation of nuclear protein complexes (Rinderle *et al.*, 1999). Furthermore, the AF10 PHD domain is reported to be a homo-oligomerization module (Linder *et al.*, 2000). Like RINGs, the PHD domain appears to function as a protein-protein interaction motif. Presumably, disruption of the structural integrity of the PHD domain by mutation of critical residues fundamentally disrupts this action. Recent studies show that some RING domains act as E3 ubiquitin ligases through interactions with the ubiquitin machinery (reviewed in Borden, 2000). Because of the similarity to the RING, we tested KAP-1 in this system. Neither the PHD alone nor the PHD-bromodomain together has detectable E3 ligase activity (data not shown). It will be necessary to test

several other PHD domains before this activity can be ruled out for the family as a whole.

Conclusions

We present the first structure of a PHD domain and show that it is an autonomously structured domain that requires zinc for folding. From sequence analysis alone it is impossible to determine how the eight conserved metal ligands are utilized to bind zinc. This uncertainty is evident from a recently modeled structure of the DNMT3L PHD domain where the ligation scheme was assumed to be LIM-like (Aapola *et al.*, 2000). The actual ligation of zinc by the PHD domain is accomplished by a cross-brace motif like that used by the RING family. Not only are the ligation schemes similar between the two domains, but also the structures are topologically identical from *ml*₁ to *ml*₆, which includes the first zinc-binding site and the anti-parallel β -strands. We identify structural determinants that distinguish ligation schemes of RING, LIM and PHD domains. In the structure of the KAP-1 PHD domain, there exists a flexible hinge region between *ml*₆ and *ml*₇ characterized by a discrete negatively charged surface. Amongst the RING domains, this same region shows structural variability, forming an extended strand or a helix. The plasticity of this region may underlie the biological specificity of these rather similar domains. The PHD domain is required for transcriptional repression by KAP-1 and we present structural determinants required for this activity, which elucidate the structural basis of disease-causing missense mutations in ATRX and ING1.

Materials and methods

Preparation of the PHD domain

A DNA fragment encoding the PHD domain of human KAP-1 (amino acids 618–679) was subcloned into the pQE30 expression vector (Qiagen) and expressed in *E. coli* BL21(DE3) cells (Novagen). The His-tagged protein contained an additional 17 N-terminal amino acids, MRGSHHHHHHSGDSIIIDE, amino acids 618–679 of KAP-1, and nine C-terminal amino acids, VDLQACKLN. For the untagged KAP-1 PHD domain protein, a DNA fragment encoding amino acids 618–679 was subcloned into the expression vector pQE50 (Qiagen) and expressed in *E. coli* SG13009 cells (Qiagen). The expressed protein contained four N-terminal amino acids, MRGS, followed by amino acids 618–679 of KAP-1. His-tagged or untagged protein was prepared from logarithmically growing bacteria cultured in either 2YT media or in minimal media, containing ^{15}N -NH $_4\text{Cl}$ as the sole nitrogen source and supplemented with 100 μM zinc acetate (for uniformly labeled ^{15}N protein). In both types of medium, protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside for 3 h at 37°C. Induced bacterial pellets were lysed in 20 mM NaH $_2$ PO $_4$ pH 7.5, 500 mM NaCl, 5 mM dithiothreitol (DTT). The His-tagged PHD domain was purified by affinity chromatography on a Ni $^{2+}$ -NTA-agarose column (Qiagen), followed by fractionation through a Superdex 75 size exclusion column (Pharmacia). The untagged PHD domain was purified sequentially by ion exchange chromatography (DEAE-cellulose), hydrophobic interaction chromatography (butyl-Sepharose) and size exclusion chromatography (Superdex 75). NMR experiments utilized both constructs, while metal binding analysis (i.e. ICP and CD) used only the untagged PHD construct. W664A, P654C and C651F mutant proteins were produced as His-tagged proteins and purified as described above.

Site-directed mutagenesis

Site-directed point mutations in the KAP-1 PHD domain were engineered by standard overlap extension PCR-mediated mutagenesis procedures. The mutagenic primers for the described mutations (Figure 6) contained the following codons: CC628,631AA, TGC to GC; C628R, TGC to CGC; V630S, GTC to AGC; G635A, GGC to GCC; D636A, GAT to GCT; M639C, ATG to TGC; C643A, TGT to GCT; C646V, TGT to GTT; F647Y, TTC to TAC; H648C, CAC to TGC; C651F, TGT to TTT; P654C, CCG to TGC; Q657E, CAG to GAG; P660A, CCA to GCA; W664A, TGG to GCG; C666A, TGC to GCC; SL667,668TF, TCA and CTC to ACA and TTC. Appropriate reading frame fusions and integrity of flanking sequences for all constructs created by PCR were confirmed by DNA sequence analysis of both strands.

Transient transfections

Protein expression from all plasmids was confirmed by transient transfection of COS-1 cells followed by immunoprecipitation of [^{35}S]methionine-labeled cell extracts. All transcription assay transfections were carried out as previously described (Ryan *et al.*, 1999).

ICP spectrometry

ICP spectrometry was conducted using the untagged PHD sample at Galbraith Laboratories, Inc. (Knoxville, TN). Twenty-five microliters of a 3.0 mM PHD sample (20 mM NaH $_2$ PO $_4$, 500 mM NaCl, 5 mM DTT pH 7.5) were prepared for ICP analysis using a wet ash digestion procedure as previously described (Bock, 1979). Measurements were made on a Perkin-Elmer P2000 using a primary wavelength of 213.856 nm (Wallace, 1981).

CD spectroscopy

Spectra were collected on a Jasco 810 spectropolarimeter at 25°C using a 1 mm path-length cuvette. For the unfolding experiments (Figure 2), the concentration of the untagged PHD sample was 30 μM in 20 mM NaH $_2$ PO $_4$, 50 mM NaCl pH 7.5. After an initial spectrum was collected, a 6-fold molar excess of EDTA was added and pH was adjusted to 7.5. The sample was then left at room temperature for 16 h to ensure it had reached equilibrium. Spectra were then recorded. For the comparison of KAP-1 PHD mutants (Figure 7), the concentration of PHD wild type and PHD mutants ranged from 15 to 30 μM in 20 mM NaH $_2$ PO $_4$, 50 mM NaCl pH 7.5 at 25°C. The buffer contribution was subtracted and signals normalized to protein concentrations.

NMR spectroscopy

For NMR, KAP-1 PHD domain protein concentration ranged from 1.5 to 3.0 mM in 20 mM NaH $_2$ PO $_4$, 500 mM NaCl, 5 mM DTT pH 7.5. No

spectral changes were observed in this sample concentration range. For ^1H NMR of KAP-1 PHD mutants (Figure 7), protein concentrations ranged from 1.1 to 2.5 mM in 20 mM NaH $_2$ PO $_4$, 500 mM NaCl, 5 mM DTT pH 7.5. Spectra were recorded at 30°C on a Bruker DRX500 spectrometer. Sequential assignments were obtained using 3D $^1\text{H}/^{15}\text{N}$ NOESY-HSQC, 3D $^1\text{H}/^{15}\text{N}$ HMQC-NOESY-HSQC and 3D $^1\text{H}/^{15}\text{N}$ total correlated spectroscopy (TOCSY)-HSQC spectra. Side-chain resonances were assigned using 3D $^1\text{H}/^{15}\text{N}$ TOCSY-HSQC, 2D ^1H -TOCSY and 2D ^1H double quantum filter (DQF)-COSY spectra. Aromatic protons were assigned through 2D ^1H -TOCSY, 2D ^1H -NOESY and 2D ^1H DQF-COSY spectra recorded in $^2\text{H}_2\text{O}$. 2D data were collected and analyzed as described (Borden *et al.*, 1995a,b). For side-chain and aromatic assignments, a construct with no His tag was used. 3D $^1\text{H}/^{15}\text{N}$ NOESY-HSQC experiments (with mixing times, τ_m , of 75 and 150 ms) and 2D ^1H -NOESY experiments (τ_m of 75 and 150 ms) were also used for obtaining interproton distance restraints. ϕ angle restraints were determined based on the $^3J_{\text{HN,H}\alpha}$ coupling constants measured in a 3D HNHA experiment. Slowly exchanging amide protons were identified from 2D ^{15}N -HSQC spectra recorded 12 h after the $^1\text{H}_2\text{O}$ buffer was exchanged for $^2\text{H}_2\text{O}$. Detailed descriptions of these experiments along with their original references have been reviewed elsewhere (Clare and Gronenborn, 1994; Cavanagh, 1996). All NMR data were processed using the NMRpipe software system (Delaglio *et al.*, 1995) and analyzed with the program NMRview (Johnson *et al.*, 1994).

Structure calculations

NOE-derived distance restraints were obtained from 3D $^1\text{H}/^{15}\text{N}$ NOESY-HSQC spectra and 2D ^1H -NOESY spectra. The intensities of NOE cross-peaks assigned in the 2D ^1H -NOESY and the 3D $^1\text{H}/^{15}\text{N}$ NOESY-HSQC spectra were classified as strong (1.8–3.0 Å), medium (1.8–4.0 Å) and weak (1.8–5.0 Å) using secondary structure elements for calibration. Pseudo-atom corrections were added to the upper distance limit when appropriate; for example, 2.4 and 1.0 Å were added to methyl and β methylene protons, respectively (Wüthrich, 1986). Dihedral ϕ angle restraints were estimated from $^3J_{\text{HN,H}\alpha}$ coupling constants obtained from a 3D HNHA spectrum. $^3J_{\text{HN,H}\alpha} < 6$ Hz were given ϕ angle restraints of $-60 \pm 40^\circ$, while $^3J_{\text{HN,H}\alpha} > 8$ Hz were given ϕ angle restraints of $-120 \pm 40^\circ$. Additionally, $^3J_{\text{HN,H}\alpha}$ coupling constants were obtained from highly digitized DQF-COSY spectrum in $^1\text{H}_2\text{O}$. Values obtained from coupling constants from the two methods were in agreement. Structures were calculated with a distance geometry/simulated annealing protocol in X-PLOR v. 3.851 (Brunger, 1996). Initial structure calculations were performed without any assumptions about zinc coordination. Initial structures indicated a cross-brace zinc ligation scheme (see Results and discussion). Subsequent structure calculations included zinc atoms with additional distance and angle constraints to maintain the tetrahedral bonding geometry of the sites and appropriate bond lengths as previously described by Neuhaus *et al.* (1992). Other alternative ligation schemes were tested but were unable to satisfy the experimentally derived constraints. Structures from these calculations were used for an automated, iterative assignment of remaining NOEs using ARIA (Nilges, 1995; Nilges *et al.*, 1997). In addition to NOE data, calculations used a total of six hydrogen bonds and 20 ϕ dihedral restraints. Quality of the final structures was assessed using PROCHECK-NMR (Laskowski *et al.*, 1996). Figures were prepared using the program PREPI. The coordinates for the KAP-1 PHD domain have been deposited in the Protein Data Bank under accession code 1FP0. Chemical shift information is available upon request.

Sequence and structure comparisons

Structural comparisons and r.m.s.d. calculations between PHD, PML (1bor) and IEEHV (1chc) were performed using the least squares fitting algorithm LSQFIT. The graphical overlays were viewed using PREPI. PREPI and LSQFIT were kindly provided by S. Islam and M. Sternberg, Imperial Cancer Research Fund. Sequence alignments were carried out using Clustal W 1.7.

Supplementary data

Supplementary data to this paper are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful for critical reading of the manuscript by Aneel Aggarwal, Graeme Carlile, Alex Kentsis, Pauline McIntosh and Larry Shapiro. We thank Lei Zeng for assistance in setting up NMR experiments. PREPI and

LSQFIT programs were a kind gift of Suhail Islam and Mike Sternberg (Imperial Cancer Research Fund). A.D.C. is supported by an NCI predoctoral training grant. D.C.S. is supported in part by the Wistar Basic Cancer Research Training Grant CA 09171 and DAMD 17-98-1-8269 grant. F.J.R. is supported in part by National Institutes of Health grants CA 52009, Core grant CA 10815, DK 49210, GM 54220, DAMD 17-96-1-6141, ACS NP-954, the Irving A.Hansen Memorial Foundation, the Mary A.Rumsey Memorial Foundation, and the Pew Scholars Program in the Biomedical Sciences. K.L.B.B. is a scholar of the Leukemia and Lymphoma Society. Financial support for K.L.B.B. was provided by National Institutes of Health grant RO1 CA 80728-01.

References

- Aapola, U. et al. (2000) Isolation and initial characterization of a novel zinc finger gene, *DNMT3L*, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family. *Genomics*, **65**, 293–298.
- Aasland, R., Gibson, T.J. and Stewart, A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.*, **20**, 56–59.
- Barlow, P.N., Luisi, B., Milner, A., Elliott, M. and Everett, R. (1994) Structure of the C3HC4 domain by ¹H-nuclear magnetic resonance spectroscopy. A new structural class of zinc-finger. *J. Mol. Biol.*, **237**, 201–211.
- Bellon, S.F., Rodgers, K.K., Schatz, D.G., Coleman, J.E. and Steitz, T.A. (1997) Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. *Nature Struct. Biol.*, **4**, 586–591.
- Bochar, D.A., Savard, J., Wang, W., Lafleur, D.W., Moore, P., Cote, J. and Shiekhattar, R. (2000) A family of chromatin remodeling factors related to Williams syndrome transcription factor. *Proc. Natl Acad. Sci. USA*, **97**, 1038–1043.
- Bock, R. (1979) *Decomposition Methods in Analytical Chemistry*. T & A Constable Ltd, Edinburgh, UK.
- Borden, K.L.B. (1998) RING fingers and B-boxes: zinc-binding protein-protein interaction domains. *Biochem. Cell Biol.*, **76**, 351–358.
- Borden, K.L.B. (2000) RING domains: master builders of macromolecular scaffolds? *J. Mol. Biol.*, **295**, 1103–1112.
- Borden, K.L.B. and Freemont, P.S. (1996) The RING finger domain: a recent example of a sequence-structure family. *Curr. Opin. Struct. Biol.*, **6**, 395–401.
- Borden, K.L.B., Boddy, M.N., Lally, J., O'Reilly, N.J., Martin, S., Howe, K., Solomon, E. and Freemont, P.S. (1995a) The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. *EMBO J.*, **14**, 1532–1541.
- Borden, K.L.B., Lally, J.M., Martin, S.R., O'Reilly, N.J., Etkin, L.D. and Freemont, P.S. (1995b) Novel topology of a zinc-binding domain from a protein involved in regulating early *Xenopus* development. *EMBO J.*, **14**, 5947–5956.
- Brehm, A., Nielsen, S.J., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. and Kouzarides, T. (1999) The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO J.*, **18**, 2449–2458.
- Brunker, A. (1996) *XPLOR (Version 3.843): A System for X-ray Crystallography and NMR*. Yale University Press, New Haven, CT.
- Cavanagh, J., Fairbrother, W.J., Palmer, A.G.I. and Skelton, N.J. (1996) *Protein NMR Spectroscopy*. Academic Press, San Diego, CA.
- Clore, G.M. and Gronenborn, A.M. (1994) Multidimensional heteronuclear nuclear magnetic resonance of proteins. *Methods Enzymol.*, **239**, 349–363.
- Dawid, I.B., Breen, J.J. and Toyama, R. (1998) LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet.*, **14**, 156–162.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR*, **6**, 277–293.
- Flores, T.P., Orengo, C.A., Moss, D.S. and Thornton, J.M. (1993) Comparison of conformational characteristics in structurally similar protein pairs. *Protein Sci.*, **2**, 1811–1826.
- Friedman, J.R., Fredericks, W.J., Jensen, D.E., Speicher, D.W., Huang, X.P., Neilson, E.G. and Rauscher, F.J., III (1996) KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev.*, **10**, 2067–2078.
- Gibbons, R.J. et al. (1997) Mutations in transcriptional regulator ATRX establish the functional significance of a PHD-like domain. *Nature Genet.*, **17**, 146–148.
- Gunduz, M., Ouchida, M., Fukushima, K., Hanafusa, H., Etani, T., Nishioka, S., Nishizaki, K. and Shimizu, K. (2000) Genomic structure of the human *ING1* gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. *Cancer Res.*, **60**, 3143–3146.
- Jacobson, S. and Pillus, L. (1999) Modifying chromatin and concepts of cancer. *Curr. Opin. Genet. Dev.*, **9**, 175–184.
- Johnson, M., Correia, J.J., Yphantis, D.A. and Halvorson, H.R. (1994) NMR VIEW—a computer program for the visualization and analysis of NMR data. *J. Biomol. NMR*, **6**, 603–614.
- Jones, M.H., Hamana, N., Nezu, J. and Shimane, M. (2000) A novel family of bromodomain genes. *Genomics*, **63**, 40–45.
- Klevit, R.E. (1991) Recognition of DNA by Cys2, His2 zinc fingers. *Science*, **253**, 1367–1393.
- Laskowski, R.A., Rullmann, J.A., MacArthur, M.W., Kaptein, R. and Thornton, J.M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR*, **8**, 477–486.
- Lechner, M.S., Begg, G.E., Speicher, D.W. and Rauscher, F.J., III (2000) Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. *Mol. Cell Biol.*, **20**, 6449–6465.
- Le Douarin, B. et al. (1995) The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.*, **14**, 2020–2033.
- Le Douarin, B., Nielsen, A.L., Garnier, J.M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996) A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. *EMBO J.*, **15**, 6701–6715.
- Linder, B., Newman, R., Jones, L.K., Debernardi, S., Young, B.D., Freemont, P., Verrijzer, C.P. and Saha, V. (2000) Biochemical analyses of the AF10 protein: the extended LAP/PHD-finger mediates oligomerisation. *J. Mol. Biol.*, **299**, 369–378.
- Lu, X., Meng, X., Morris, C.A. and Keating, M.T. (1998) A novel human gene, *WSTF*, is deleted in Williams syndrome. *Genomics*, **54**, 241–249.
- Neuhaus, D., Nakaseko, Y., Schwabe, J.W. and Klug, A. (1992) Solution structures of two zinc-finger domains from SWI5 obtained using two-dimensional ¹H nuclear magnetic resonance spectroscopy. A zinc-finger structure with a third strand of β-sheet. *J. Mol. Biol.*, **228**, 637–651.
- Nicholls, A., Sharp, K.A. and Honig, B. (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins*, **11**, 281–296.
- Nilges, M. (1995) Calculation of protein structures with ambiguous distance restraints. Automated assignment of ambiguous NOE crosspeaks and disulphide connectivities. *J. Mol. Biol.*, **245**, 645–660.
- Nilges, M., Macias, M.J., O'Donoghue, S.I. and Oschkinat, H. (1997) Automated NOESY interpretation with ambiguous distance restraints: the refined NMR solution structure of the pleckstrin homology domain from β-spectrin. *J. Mol. Biol.*, **269**, 408–422.
- Omichinski, J.G., Clore, G.M., Schaad, O., Felsenfeld, G., Trainor, C., Appella, E., Stahl, S.J. and Gronenborn, A.M. (1993) NMR structure of a specific DNA complex of Zn-containing DNA binding domain of GATA-1. *Science*, **261**, 438–446.
- Peng, H., Begg, G.E., Harper, S.L., Friedman, J.R., Speicher, D.W. and Rauscher, F.J., III (2000a) Biochemical analysis of the Kruppel-associated box (KRAB) transcriptional repression domain. *J. Biol. Chem.*, **275**, 18000–18010.
- Peng, H., Begg, G.E., Schultz, D.C., Friedman, J.R., Jensen, D.E., Speicher, D.W. and Rauscher, F.J., III (2000b) Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. *J. Mol. Biol.*, **295**, 1139–1162.
- Rinderle, C., Christensen, H.M., Schweiger, S., Lehrach, H. and Yaspo, M.L. (1999) AIRE encodes a nuclear protein co-localizing with cytoskeletal filaments: altered sub-cellular distribution of mutants lacking the PHD zinc fingers. *Hum. Mol. Genet.*, **8**, 277–290.
- Ryan, R.F., Schultz, D.C., Ayyanathan, K., Singh, P.B., Friedman, J.R., Fredericks, W.J. and Rauscher, F.J., III (1999) KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell Biol.*, **19**, 4366–4378.

- The Finnish-German Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy Consortium (1997) An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. The Finnish-German APECED Consortium. Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy. *Nature Genet.*, **17**, 399-403.
- Venturini, L. *et al.* (1999) TIF1 γ , a novel member of the transcriptional intermediary factor 1 family. *Oncogene*, **18**, 1209-1217.
- Wade, P.A., Jones, P.L., Vermaak, D. and Wolffe, A.P. (1998) A multiple subunit Mi-2 histone deacetylase from *Xenopus laevis* cofractionates with an associated Snf2 superfamily ATPase. *Curr. Biol.*, **8**, 843-846.
- Wallace, G. (1981) *Analytical Methods for Inductively Coupled Spectrometry*. Perkin Elmer Corp., Norwalk, CT.
- Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*. Wiley, New York, NY.

Received August 29, 2000; revised and accepted November 17, 2000

Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2 α subunit of NuRD

David C. Schultz, Josh R. Friedman, and Frank J. Rauscher III

The Wistar Institute, Philadelphia, Pennsylvania 19104, USA

Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2 α subunit of NuRD

David C. Schultz, Josh R. Friedman, and Frank J. Rauscher III¹

The Wistar Institute, Philadelphia, Pennsylvania 19104, USA

Macromolecular complexes containing histone deacetylase and ATPase activities regulate chromatin dynamics and are vitally responsible for transcriptional gene silencing in eukaryotes. The mechanisms that target these assemblies to specific loci are not as well understood. We show that the corepressor KAP-1, via its PHD (plant homeodomain) and bromodomain, links the superfamily of Krüppel associated box (KRAB) zinc finger proteins (ZFP) to the NuRD complex. We demonstrate that the tandem PHD finger and bromodomain of KAP-1, an arrangement often found in cofactor proteins but functionally ill-defined, form a cooperative unit that is required for transcriptional repression. Substitution of highly related PHD fingers or bromodomains failed to restore repression activity, suggesting high specificity in their cooperative function. Moreover, single amino acid substitutions in either the bromodomain or PHD finger, including ones that mimic disease-causing mutations in the hATR_X PHD finger, abolish repression. A search for effectors of this repression function yielded a novel isoform of the Mi-2 α protein, an integral component of the NuRD complex. Endogenous KAP-1 is associated with Mi-2 α and other components of NuRD, and KAP-1-mediated silencing requires association with NuRD and HDAC activity. These data suggest the KRAB-ZFP superfamily of repressors functions to target the histone deacetylase and chromatin remodeling activities of the NuRD complex to specific gene promoters *in vivo*.

[Key Words: KRAB-ZFPs; repression; PHD finger; bromodomain; NuRD; ATR_X]

Received November 22, 2000; revised version accepted December 21, 2000.

The complement of transcription factors organized at a gene promoter integrates different transcriptional regulatory signals that orchestrate cellular responses such as proliferation, differentiation, and apoptosis. Regulation of RNA polymerase II involves a complex interplay between DNA-protein interactions and protein-protein interactions. Whereas the general transcription factors regulate the accurate initiation of transcription, proteins that bind gene-specific DNA elements are instrumental in either negatively or positively regulating the rate of transcription (Orphanides et al. 1996; Hampsey and Reinberg 1999). Studies aimed at understanding the mechanisms of transcriptional regulation by site-specific factors have been aided by the realization that these proteins are highly modular in architecture. In addition to sequence-specific DNA binding domains, these proteins

possess an independent, functionally separable effector domain that can either activate or repress transcription. The affects of sequence-specific transcription factors in the regulation of transcription have been greatly facilitated by the identification of cofactors with which they bind and the intrinsic or associated biochemical activities of these proteins. It is clear from this strategy that these proteins possess enzymatic activities which either utilize ATP to remodel chromatin structure or covalently modify the amino-terminal tails of the core histones, creating a biochemical code that regulates gene transcription of chromatin templates (Tyler and Kadonaga 1999; Strahl and Allis 2000). Thus, sequence-specific repressor-corepressor complexes likely serve as scaffolds for the assembly of macromolecular complexes which integrate diverse transcription regulatory signals.

The Krüppel associated box (KRAB) domain is one example of an abundant amino acid sequence motif found at the N terminus of nearly one-third of all Krüppel/TFIIA-type C2H2 zinc finger proteins (Bellefroid et al. 1991). This highly conserved domain displays potent,

¹Corresponding author.

E-MAIL Rauscher@wistar.upenn.edu; FAX (215) 898-3929.

Article and publication are at www.genesdev.org/cgi/doi/10.1101/gad.869501.

DNA-binding dependent repression of transcription that requires the KAP-1 corepressor (Margolin et al. 1994; Friedman et al. 1996; Kim et al. 1996; Moosmann et al. 1996, 1997). The primary amino acid sequence of KAP-1 revealed the presence of several conserved signature motifs, including a RING finger, B boxes, and a coiled-coil region (RBCC), which collectively form an integrated domain that is both necessary and sufficient to directly interact with the KRAB domain (Fig. 1A) (Peng et al. 2000a,b). The C-terminal sequence of KAP-1 revealed a conserved PHD (plant homeodomain) finger and bromodomain (Fig. 1A). This particular spatial arrangement of motifs has defined an emerging family of transcriptional regulators that include TIF1 α and TIF1 γ (Fig. 1A) (Le Douarin et al. 1995; Venturini et al. 1999). All members of this protein family have been shown to repress transcription when tethered to DNA, and the mechanisms by which they repress transcription are currently being defined. In this aspect, a direct interaction between KAP-1 and mammalian members of the nonhistone chromosomal protein, heterochromatin protein 1 (HP1) has been described (Le Douarin et al. 1996; Nielsen et al. 1999; Ryan et al. 1999; Lechner et al. 2000). Biochemical studies indicate that this interaction is dependent upon the chromoshadow domain of HP1 proteins and a core, hydrophobic pentapeptide sequence (PxVxL) in KAP-1 (Lechner et al. 2000). Furthermore, indirect immunofluorescence studies of interphase nuclei indicated that KAP-1 colocalizes with both heterochromatic and euchromatic HP1 proteins (Ryan et al. 1999). These particular studies suggest that the KRAB-KAP-1 complex may mediate repression in part via the maintenance or initiation of heterochromatic chromosomal environments. Moreover, it remains to be determined what role the PHD finger and bromodomain possess in KAP-1 repression, or if these domains integrate additional factors which facilitate HP1 recruitment and function.

The PHD finger and bromodomain are two highly conserved protein motifs found in an increasing number of proteins with roles in regulating transcription via modification of chromatin structure (Aasland et al. 1995; Jeanmougin et al. 1997; Winston and Allis 1999). Furthermore, these two motifs are commonly linked architecturally in a number of proteins (Le Douarin et al. 1995, 1996; Friedman et al. 1996; Venturini et al. 1999; Bochar et al. 2000; Jones et al. 2000a,b). Although a specific function for the PHD finger remains to be fully described, naturally occurring single amino acid substitutions in the PHD finger of the hATRAX and AIRE1 proteins predispose individuals to α -thalassemia, developmental defects, and autoimmune disease, respectively (Gibbons et al. 1997; Bjorses et al. 2000). These observations emphasize the biological importance of this domain in the functions of these proteins. On the other hand, structural and biochemical studies of three independent bromodomains clearly establish a role for this domain in the molecular recognition of the N termini of acetylated histone tails (Dhalluin et al. 1999; Jacobson et al. 2000; Owen et al. 2000). Moreover, mutations that specifically disrupt the bromodomain/acetyl-histone in-

teraction have been shown to functionally impair the transcriptional regulatory activities of these proteins in vivo (Cairns et al. 1999; Sterner et al. 1999; Syntichaki et al. 2000).

To understand the molecular role of the PHD finger and bromodomain in KAP-1-mediated transcriptional repression, we have used a biochemical approach to identify effector functions of this bipartite domain. Our data suggest that the spatial conservation of these motifs is essential for optimal protein function. These data are consistent with the PHD finger and bromodomain providing an interface for protein-protein interactions, and therefore may have broad implications in understanding the function of proteins that maintain this particular spatial arrangement of motifs. Comparative analyses of proteins containing these two motifs indicate that this bipartite domain is not functionally equivalent among all proteins. Thus, it is likely that the function of this integrated domain will be dependent upon those associated proteins that specifically interact with KAP-1 sequences. The interaction of KAP-1 with Mi-2 α is consistent with this hypothesis and implies a role for histone deacetylases in transcriptional repression mediated by the KRAB-ZFP-KAP-1 repressor/corepressor system. Moreover, the estimated 300 to 700 KRAB zinc finger proteins in the human proteome would serve as an effective sequence-specific targeting mechanism for the histone deacetylase complex NuRD to gene promoters in vivo.

Results

Several pieces of evidence indicate that KAP-1 functions as a transcriptional corepressor for the KRAB repression module, including an intrinsic ability to repress transcription when directly tethered to DNA templates (Friedman et al. 1996; Moosmann et al. 1996; Agata et al. 1999; Nielsen et al. 1999; Ryan et al. 1999). To identify regions of KAP-1 which are required for its transcriptional repression function, we have engineered a series of KAP-1 deletion mutants with the heterologous GAL4 DNA binding domain to grossly map repression domains (Fig. 1B). Each heterologous fusion protein was tested for stable expression and nuclear localization in COS1 cells (Fig. 1C; data not shown). Nuclear extracts prepared from COS1 cells transfected with each construct demonstrated GAL4 DNA binding activity in vitro (data not shown). In general, amino acids C-terminal to the RBCC region define at least two independent repression domains within KAP-1 that appear to be additive in nature in order to obtain maximal levels of transcriptional repression. One of these domains includes the recently defined HP1 binding domain of KAP-1 (Ryan et al. 1999; Lechner et al. 2000). Optimal repression of transcription was dependent upon the PHD finger and bromodomain. When autonomously tethered to DNA, these two motifs demonstrated a significant level of repression, suggesting that they can function as an independent repression domain (Fig. 1B). This repression activity was DNA binding-dependent, dose-dependent, and universal for all pro-

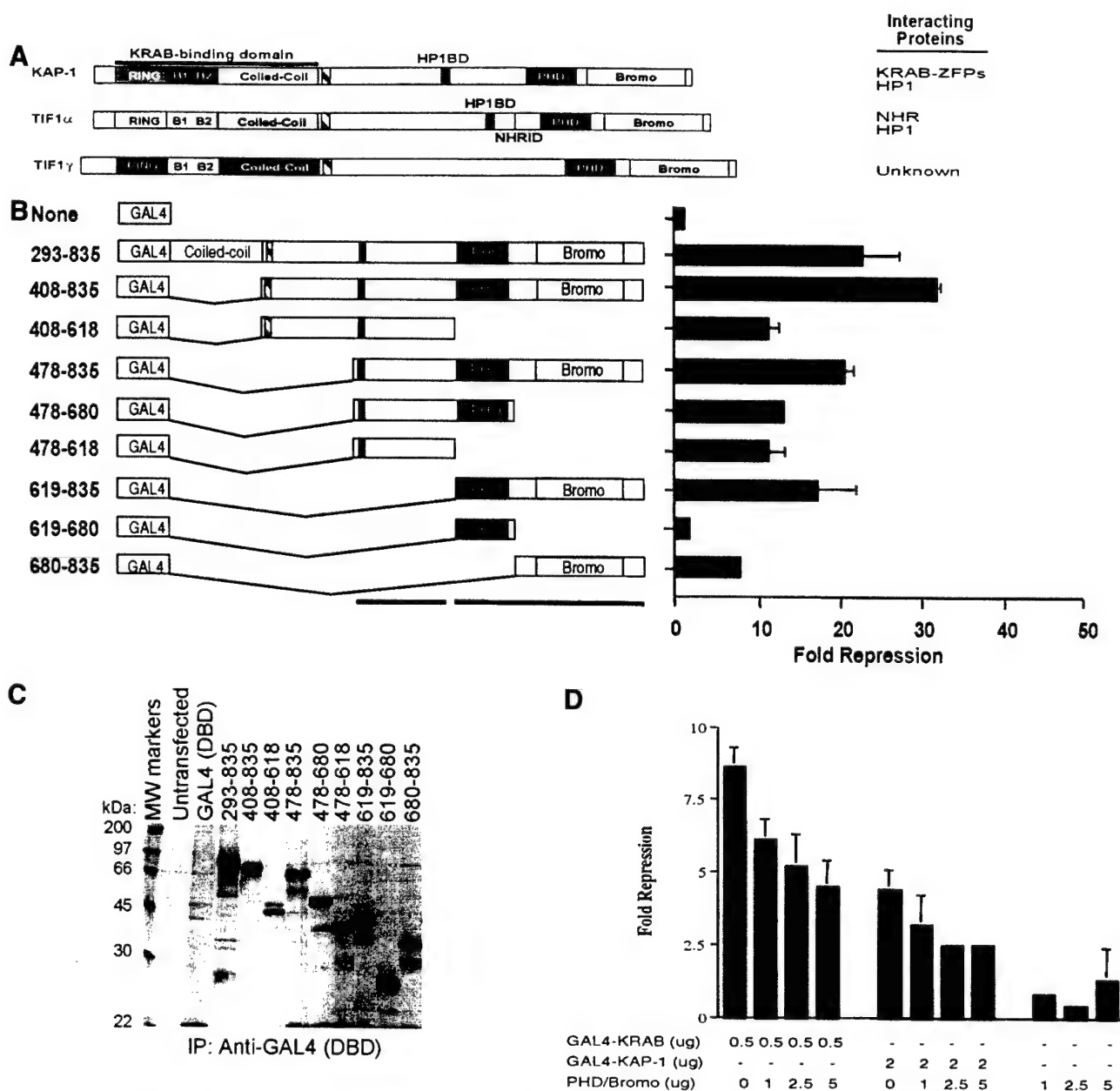


Figure 1. Analysis of the intrinsic repression activity of KAP-1 indicates that the PHD finger and bromodomain are required and sufficient to repress transcription. (A) Schematic illustration of the KAP-1/TIF1 family of transcriptional regulators. The KRAB binding domain in KAP-1 is indicated. (Striped box) TIF signature sequence (TSS), (black box) HP1 binding domain (HP1BD), (thin black line) nuclear hormone receptor interaction domain. (B) Schematic illustration of the heterologous GAL4-KAP-1 constructs, as defined by the amino acid numbers at the left. The intrinsic repressor activity of KAP-1 was measured in a transient assay, using a minimal TK-luciferase reporter template regulated by five consensus GAL4 UAS. All experiments were done in NIH/3T3 cells with 0.5 μ g of reporter plasmid and 5 μ g of the indicated heterologous GAL4-KAP-1 construct. Fold repression represents the ratio of luciferase activity measured for the reporter alone to the activity measured in the presence of the indicated effector proteins after normalization for transfection efficiency. Error bars represent the standard deviation for three independent transfections. Absence of error bars indicates a standard deviation too small to physically illustrate. (C) Each plasmid was tested for stable protein expression via transfection into COS1 cells followed by immunoprecipitation of [35 S]methionine-labeled whole cell extracts with anti-GAL4 (DBD) IgG (1 μ g). (D) Overexpression of the KAP-1 PHD finger and bromodomain (amino acids 619 to 835) demonstrated a dose-dependent dominant effect on GAL4-KRAB- and GAL4-KAP-1-mediated transcriptional repression. Transcriptional effects were monitored in a transient assay in which 0.5 μ g of the 5x-GAL4 UAS-SV40 luciferase reporter and the indicated amounts of expression plasmids transfected into NIH/3T3 cells. Fold repression was calculated as described. Expression of the dominant negative protein was confirmed in COS1 cells (data not shown).

motors tested, including the SV40 immediate early enhancer, the adenovirus major late promoter, and a minimal genomic promoter fragment of the DNA polymerase β gene (data not shown). Neither the PHD finger nor the bromodomain could independently recapitulate this repression activity, indicating that these two motifs function cooperatively to repress transcription (Fig. 1B). Furthermore, expression of the segment encoding the PHD finger and bromodomain of KAP-1 relieved both GAL4-KRAB and GAL4-KAP-1 directed repression, suggesting that repression by this bipartite domain is mediated by at least one or more titratable effector molecules (Fig. 1D). The combination of these data suggests that the PHD finger and bromodomain of KAP-1 function as an integrated unit to facilitate repression of transcription by KAP-1. Moreover, these data implicate an additional repression mechanism that is independent of the recruitment of HP1 proteins (Ryan et al. 1999; Lechner et al. 2000).

Comparative analysis of the KAP-1 PHD finger and bromodomain with domains from related proteins

Because the KAP-1 orthologs, TIF1 α and TIF1 γ , have been reported to function as transcriptional repressors, we compared the repression properties of the PHD finger and bromodomain of all three family members. To directly test this hypothesis, we have constructed heterologous fusions between the GAL4 DNA binding domain and the PHD finger and bromodomains of TIF1 α , TIF1 γ , and WCRF180 (Bochar et al. 2000) (Fig. 2A). In addition, we engineered chimeric proteins in which either the PHD finger or bromodomain of TIF1 α , TIF1 γ , hATRX, Mi-2 α , WCRF180, or hGCN5 were substituted for the KAP-1 sequences in order to address the specificity of the repression activity displayed by this bipartite domain (Fig. 2A). Stable expression and nuclear localization of each chimeric fusion protein were confirmed in COS1 cells (Fig. 2B and data not shown). Nuclear ex-

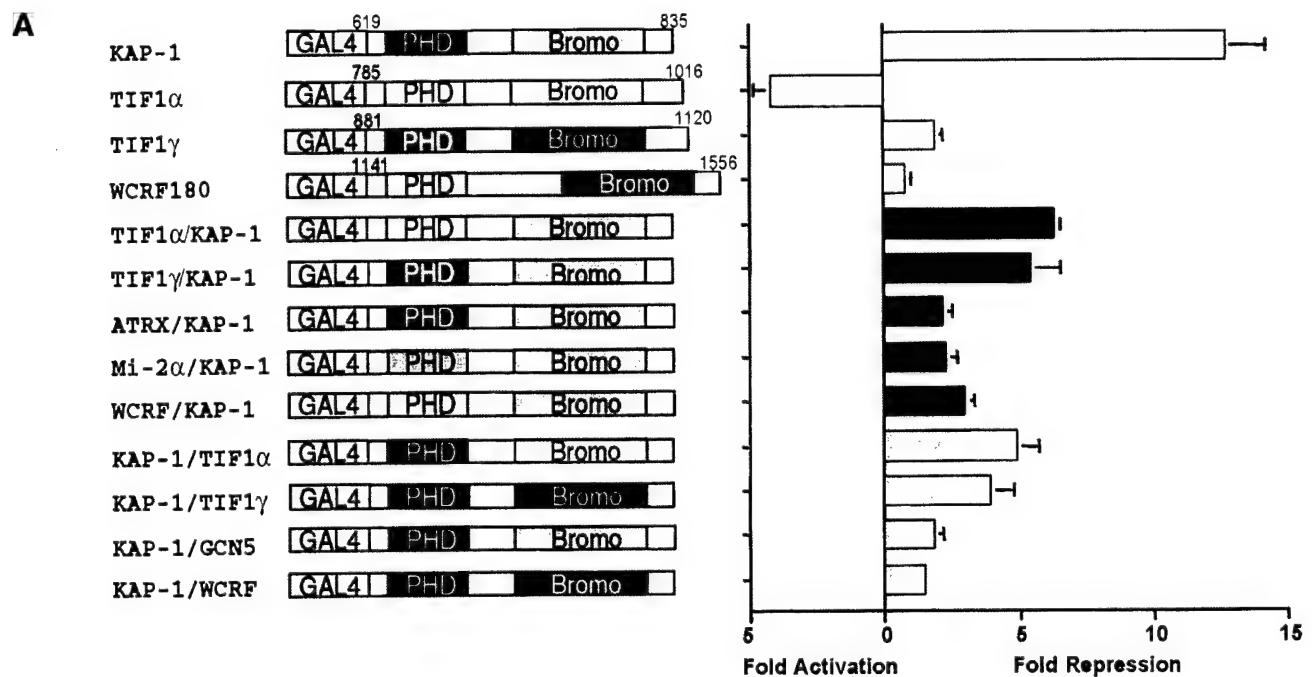


Figure 2. Heterologous chimeric fusions of related PHD fingers or bromodomains to KAP-1. (A) Schematic illustration of chimeric fusion proteins engineered. The chimeric fusion proteins possess the following amino acid sequences of the individual proteins, respectively: TIF1 α /KAP-1 (815–876/680–835), TIF1 γ /KAP-1 (880–941/680–835), ATRX/KAP-1 (210–283/680–835), Mi-2 α /KAP-1 (372–423/680–835), WCRF180/KAP-1 (1141–1205/680–835), KAP-1/TIF1 α (619–679/877–1016), KAP-1/TIF1 γ (619–679/942–1120), KAP-1/GCN5 (619–679/714–837), and KAP-1/WCRF180 (619–679/1389–1556). All experiments were done in NIH/3T3 cells, as described in Fig. 1. (White bars) Natural PHD finger/bromodomain fusions, (black bars) chimeras with the KAP-1 bromodomain, (gray bars) chimeras with the KAP-1 PHD finger. (B) Each plasmid was tested for stable protein expression via transfection into COS1 cells followed by immunoprecipitation of [35 S]methionine-labeled whole cell extracts with anti-GAL4 (DBD) IgG (1 μ g). (Arrow) the migration of the heterologous GAL4–WCRF180 PHD finger/bromodomain, (asterisk) a nonspecific band retained during the immunoprecipitation.

munoprecipitation of [35 S]methionine-labeled whole cell extracts with anti-GAL4 (DBD) IgG (1 μ g). (Arrow) the migration of the heterologous GAL4–WCRF180 PHD finger/bromodomain, (asterisk) a nonspecific band retained during the immunoprecipitation.

tracts prepared from COS1 cells transfected with each construct demonstrated GAL4 DNA binding activity *in vitro* (data not shown). When tethered to DNA, the PHD finger and bromodomain of TIF1 α demonstrated a potential to activate transcription of the reporter template, whereas the PHD finger and bromodomain of TIF1 γ and WCRF180 each failed to demonstrate any appreciable potentiation of transcription (Fig. 2A). Heterologous substitution of the KAP-1 PHD finger or bromodomain with similar sequences from other transcriptional regulators failed to completely complement the repression activity of the wild-type KAP-1 PHD finger and bromodomain (Fig. 2A). Interestingly, when the bromodomain of KAP-1 was tethered with the PHD finger of TIF1 α , the chimeric protein functioned to repress transcription from the reporter template. In general, the KAP-1 bromodomain, when fused to any PHD finger, slightly enhanced the repression potential of the chimeric protein, suggesting that the bromodomain of KAP-1 is essential in mediating specific interactions with effectors that promote repression of transcription (Fig. 2A). The combination of these data provides additional support that the PHD finger and bromodomain of KAP-1 function as an integrated functional unit to specifically repress transcription. The data also imply that not all PHD fingers and bromodomains are functionally equivalent, despite demonstrating homology in the primary amino acid sequence. Furthermore, the spatial conservation of these two motifs architecturally in proteins is not sufficient to infer a common function for this bipartite domain.

Mutational analysis of the KAP-1 PHD finger and bromodomain in repression

In order to correlate the molecular relationship between amino acids in the PHD finger and bromodomain and transcriptional repression, we have used a site-directed mutagenesis approach. All amino acid substitutions were made in the context of the heterologous GAL4-PHD finger/bromodomain fusion protein. Stable expression and nuclear localization of all heterologous fusion proteins were confirmed in COS1 cells (Fig. 3C and data not shown). Nuclear extracts prepared from COS1 cells transfected with each construct demonstrated GAL4 DNA binding activity *in vitro* (data not shown). The effect of each mutation on the repression properties of this bipartite domain was determined in DNA-template based assays. As an initial strategy, we have targeted amino acid residues in the PHD finger of KAP-1 (C628R, V630S, C651F, P654C, and Q657E; Fig. 3A) which spatially parallel the position of naturally occurring mutations in hATRX that confer an inherited susceptibility to developmental defects (Gibbons et al. 1997). As illustrated in Figure 3D, the C628R and C651F mutations significantly impaired the repression activity of this domain, whereas the P654C mutation demonstrated an intermediate effect. Each of these three amino acids is highly conserved in the core consensus sequence of the PHD finger (Fig. 3A). The solution structure of the KAP-1 PHD finger indicates that these amino acids bind

zinc in a cross-brace topology between anti-parallel β -strands reminiscent of RING domains (Capili et al. 2001). Both C628 and C651 are critical amino acids in the coordination of zinc that forms Site I. Chemical and physical characterization of the C651F mutation in the KAP-1 PHD finger results in an unstructured protein. The P654 residue is positioned two amino acids downstream from metal ligand-6, which completes site I zinc chelation. Introduction of an extra cysteine at this position may introduce a new metal ligand. Indeed, the chemical and physical properties of this mutant protein are consistent with an unstructured protein. The V630S and Q657E mutations had minimal effects on repression. This effect may not be entirely surprising, as these amino acids are less conserved and therefore represent residues that mediate context-specific interactions. In addition to these disease-related mutations, we have systematically analyzed the role of both conserved and non-conserved amino acid residues of the PHD finger (Fig. 3A) in KAP-1-mediated transcriptional repression (Capili et al. 2001). In general, the ability of the KAP-1 PHD domain to bind zinc and maintain the hydrophobic core are both required for its proper folding and ability to function in transcriptional repression.

We also used the information provided by the structure of the pCAF bromodomain to design six independent mutations in each of the four α -helices and one mutation in the variable loop region between helix Z and helix A of the KAP-1 bromodomain (Fig. 3B). As illustrated in Figure 3D, mutations in helix B (F761A) and helix C (DD 778,781AA, trunc 781, and F791E) significantly impaired transcriptional repression. The S730I mutation in the variable loop region between helix Z and helix A also displayed an intermediate effect on the repression potential of this domain, reaffirming the potential role for this region in macromolecular recognition (Fig. 3D). Overall, the data from this mutational analysis are consistent with the PHD finger and bromodomain of KAP-1 functioning cooperatively, as independent mutations in either domain can significantly disrupt the intrinsic repression potential of this bipartite domain. One interpretation of these data is that these two motifs provide an interface for protein-protein interactions with downstream effectors of transcriptional repression.

Identification of downstream effectors of KAP-1 PHD/bromodomain-mediated transcriptional repression

To identify potential mechanisms of transcriptional repression directed by the KAP-1 PHD finger and bromodomain, a heterologous fusion between the LEXA DNA binding domain and the PHD finger and bromodomain of KAP-1 was used as bait in a yeast two-hybrid screen. To confirm the specificity of any potential protein interactions identified in this screen, we utilized several of the amino acid substitutions engineered for the structure/function analysis of this bipartite domain. Appropriate expression of the heterologous fusion proteins was confirmed by Western blot analysis, and bait proteins dis-

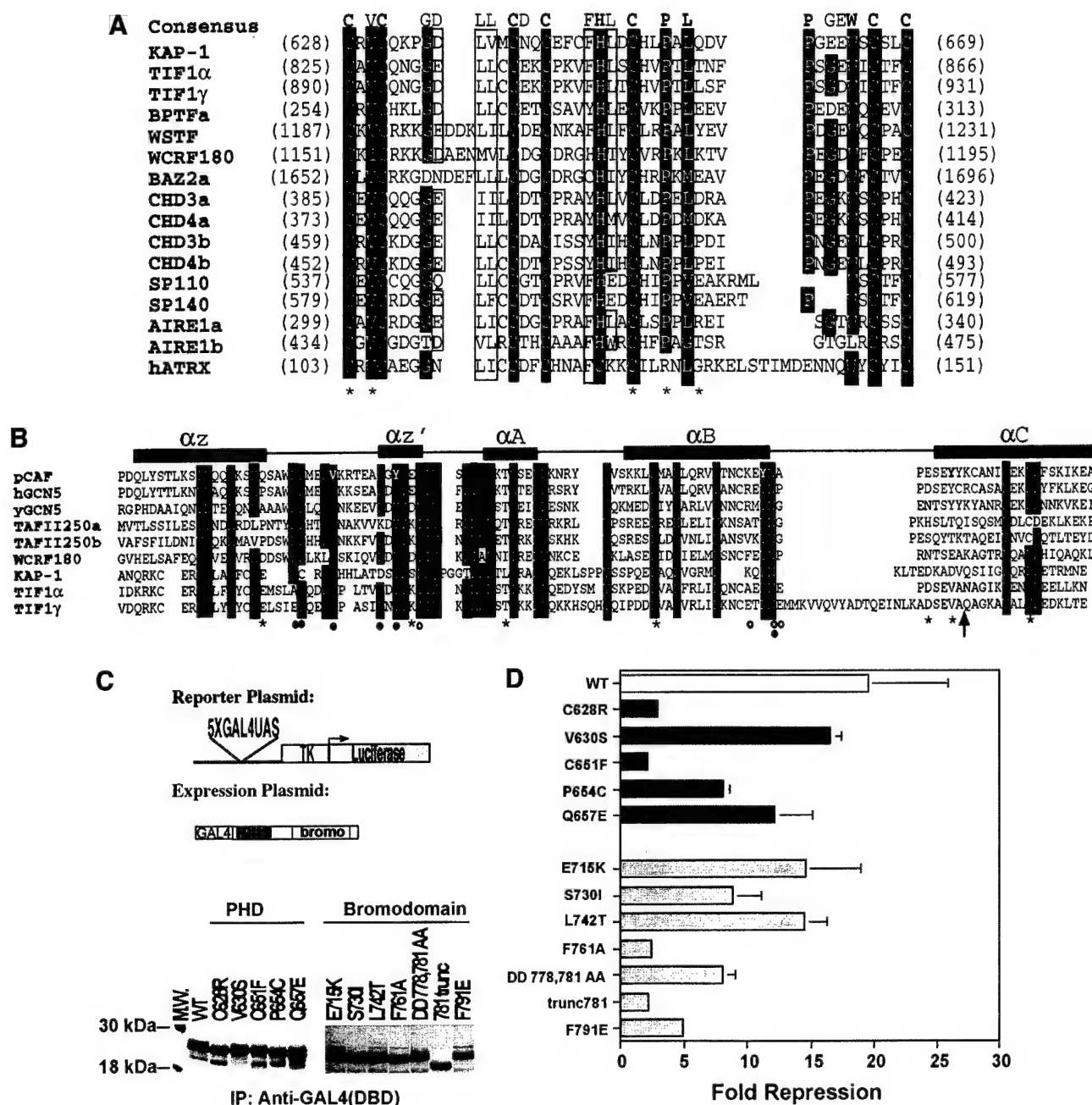


Figure 3. Mutations in the PHD finger and bromodomain significantly impair the intrinsic repression activity of this integrated transcriptional repression domain. (A) Amino acid sequence alignment of the KAP-1 PHD finger with related sequences from 15 independent proteins. The numbers in brackets indicate the corresponding amino acids in each protein. Strictly, conserved amino acids are shaded in black. Boxed amino acids are those in which the chemical nature of the side chain has been conserved. Asterisks indicate amino acids that were mutated to match naturally occurring mutations in the hATRX protein (Gibbons et al. 1997). Each mutation was made in the context of the GAL4-KAP-1 (619–835) expression construct. (B) Amino acid sequence alignment of the KAP-1 bromodomain with related sequences from TIF1 α , TIF1 γ , WCRF180, hGCN5, yGCN5, hTAFII250, and pCAF. Schematic illustration of the secondary structure and relevant position of each structural element in the bromodomain sequence are indicated above. Amino acid residues that have been conserved or where the chemical nature of the side chain has been maintained are shaded in black. Asterisks mark the amino acids that were mutated in this study. The arrow identifies the position of the 781trunc mutation. Each mutation was made in the context of the GAL4-KAP-1 (619–835) expression construct. Bolded amino acids in the pCAF sequence are those mutated in the study by Dhaliun et al. (1999). (Filled circles) Amino acids in the crystal structure that contact the acetyl moiety of acetylated histones (Owen et al. 2000). (Open circles) Amino acids in the crystal structure that contact the amide backbone of histone H4 (Owen et al. 2000). (C) Schematic diagram of the luciferase reporter and the heterologous GAL4-effector plasmid. Stable expression of each protein was determined via transfection into COS1 cells followed by immunoprecipitation of [³⁵S]methionine-labeled whole cell extracts with anti-GAL4 (DBD) IgG (1 μ g). (D) All experiments were done in NIH/3T3 cells, as described in Fig. 1. (Black bars) PHD finger mutations, (gray bars) bromodomain mutations.

played no ability to self-activate the selection promoters (data not shown).

From 40 million library transformants, four different gene products were recovered based on coactivation of both the integrated LEXA-responsive *His3* and *LacZ* reporter genes. The nucleotide sequence of three independent transformants of one gene product indicated that each clone possessed an identical fusion junction to the GAL4 activation domain and that the sequence was identical to the 3' nucleotide sequence of the dermatomyositis-specific autoantigen, Mi-2 α /CHD3. Upon reintroduction into yeast, this GAL4 activation domain fusion protein displayed robust ability to activate the *LacZ* reporter with the wild-type LEXA-KAP-1 fusion protein (Fig. 4A), but failed to activate the reporter with other irrelevant test baits (data not shown). Two PHD finger mutants (V630S and G635A) that demonstrated nearly wild-type levels of transcriptional repression activity also displayed the ability to activate the *LacZ* reporter (Fig. 4A). In contrast, PHD finger mutants CC628, 631AA, C651F, and W664A, which possess impaired repression activity, either failed to activate these reporters or activated with reduced efficiency (Fig. 4A). This putative protein-protein interaction was also observed to be dependent on an intact bromodomain, as the 781 truncation mutation displayed reduced affinity for the Mi-2 α in the two-hybrid assay (Fig. 4A). These observations are completely consistent with the transcriptional effects observed in transient transfection assays (Fig. 3D) in which the repression activity was dependent upon both domains. Furthermore, mutations in either the PHD finger or the bromodomain were independently sufficient to ablate the repression function of this bipartite domain. The combination of these results strengthens the argument that the PHD finger and bromodomain of KAP-1 function as an integrated functional unit which provides an interface for protein-protein interactions with molecules that facilitate repression by the KRAB-KAP-1 complex.

Mi-2 α /CHD3 is a member of the CHD family of proteins which were named based on the possession of several signature motifs, that is, chromodomains, ATPase/helicases, and DNA binding modules [Delmas et al. 1993; Woodage et al. 1997]. Mi-2 α /CHD3 is closely related to the Mi-2 β /CHD4 protein. Comparison of the amino acids in the putative KAP-1 interaction domain of Mi-2 α /CHD3 with Mi-2 β /CHD4 revealed that these two proteins are 80% identical, yet all three KAP-1 interacting clones rescued in this screen possessed sequence specific to Mi-2 α /CHD3. Based on the sequences of these clones, amino acids 1686 to 2000 harbor the minimal KAP-1 interaction domain (KID) [Fig. 4B]. This sequence encodes a protein with an additional 30 amino acids at the C terminus of the protein that are not present in the putative protein described by Woodage et al. (1997) for CHD3 (accession no. AF006515), yet the remainders of the two sequences were identical (Fig. 4C). To test the functional significance of the novel 30 amino acids contained in our rescued clones versus the C-terminal sequence reported for CHD3, and the ability of Mi-2 β /

CHD4 to interact with KAP-1, we engineered fusions of these C-terminal sequences with the GAL4 activation domain, respectively (Fig. 4B). Upon introduction into yeast, the wild-type LEXA-PHD/bromodomain fusion of KAP-1 displayed no activation of the *LacZ* reporter for either protein, indicating that the C termini of these two proteins, CHD3 and Mi-2 β /CHD4, are not functionally equivalent to the Mi-2 α sequence we rescued in the two-hybrid screen (Fig. 4B). Closer inspection of the C-terminal amino acid residues revealed that Mi-2 α /CHD3 quickly diverges from Mi-2 β /CHD4 starting at amino acid 1909 and continues for an additional 91 novel amino acids (Fig. 4C). It is these amino acid residues that most likely confer the specificity of the Mi-2 α /CHD3 interaction with KAP-1.

The Mi-2 family of proteins has been described as an integral component of a high molecular weight multiprotein complex containing histone deacetylase activity [Tong et al. 1998; Wade et al. 1998; Xue et al. 1998; Zhang et al. 1998a; Kim et al. 1999]. We have generated polyclonal antibodies against a unique antigen in Mi-2 α (amino acids 1515 to 1708, accession no. 3298562). Western blot analysis of conventionally purified NuRD from a 0.5 M phosphocellulose fractionated HeLa nuclear extract with these Mi-2 α -specific antibodies demonstrated little immunoreactivity (Fig. 5A,B), consistent with previous observations that no peptide sequences specific for Mi-2 α were identified in these purifications. Since all the peptide sequences reported for the NuRD/NRD complex were specific for Mi-2 β , this observation suggested that Mi-2 α might exist in an independent protein complex. Western blot analysis of phosphocellulose fractionated HeLa nuclear extract revealed that the majority of Mi-2 α eluted in the 1.0 M fraction, while Mi-2 β elutes evenly between the 0.5 M and 1.0 M fractions. KAP-1 is an abundant nuclear protein that demonstrated near equal elution in all phosphocellulose fractions (Fig. 5A).

To verify an interaction in vivo between KAP-1 and Mi-2 α , we used coimmunoprecipitation experiments of endogenous proteins from the 1.0 M phosphocellulose fraction to maximize the chance to observe an interaction between these two proteins. Western blot analysis of Mi-2 α immunoprecipitates revealed the retention of both HDAC1 and RbAp48, indicating that Mi-2 α likely exists in some NuRD-like complex (Fig. 5B). Two independent antibodies against nonoverlapping antigens of KAP-1 specifically coprecipitated Mi-2 α , HDAC1, and RbAp48 (Fig. 5B). None of these polypeptides was observed in immunoprecipitates with preimmune IgG, and no immunoreactivity for Mi-2 β was observed in these immunoprecipitates, consistent with our observation in the two-hybrid screen. This result demonstrates an association between KAP-1 and Mi-2 α in vivo. The significance of the association of KAP-1 exclusively with the faster migrating species in the Mi-2 α immunoprecipitates is unclear at this time. Although this species is not abundant in the input, it does represent a major form of Mi-2 α in the self-immunoprecipitate and may represent a degradation product. Furthermore, it is possible that this protein represents an alternatively spliced transcript

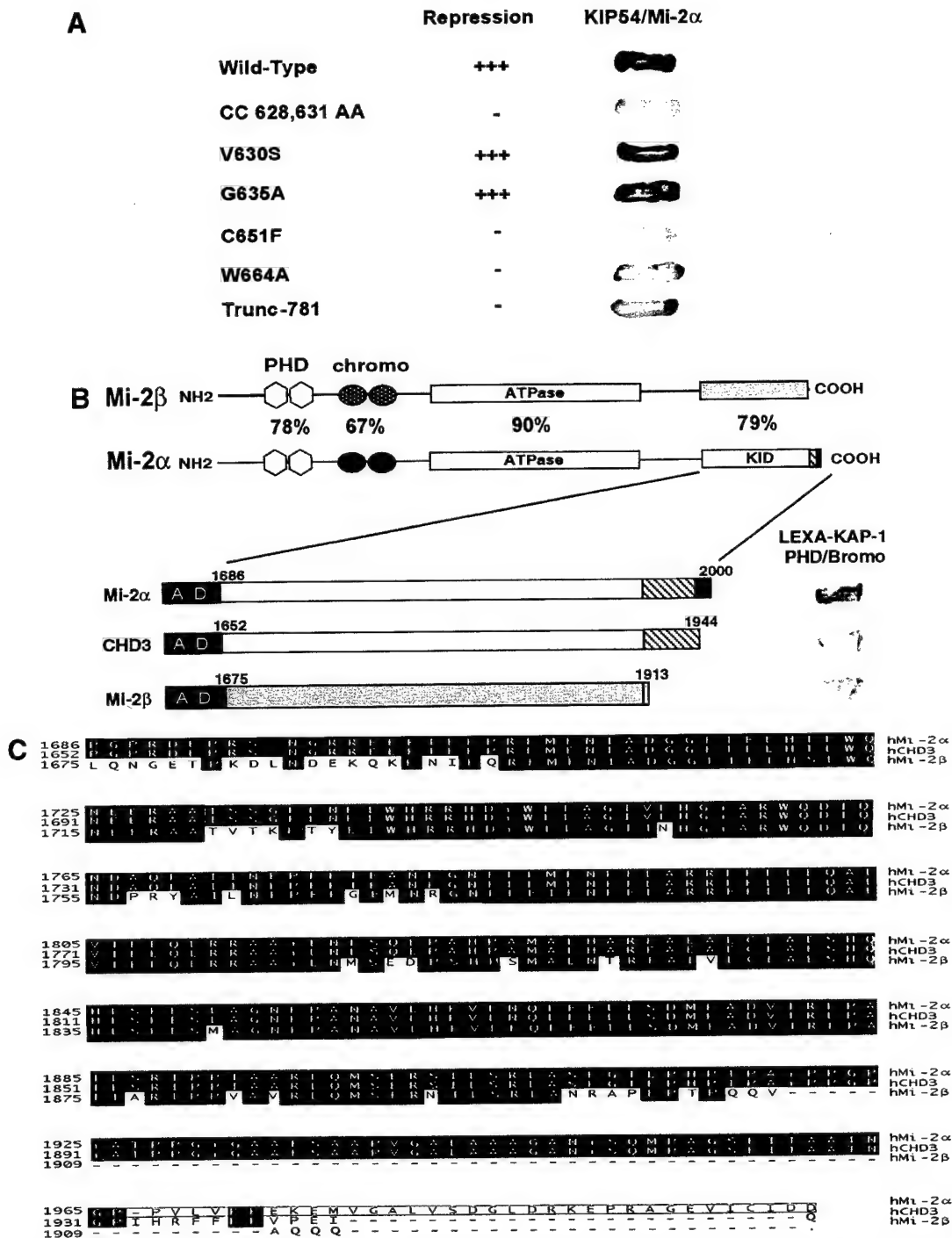


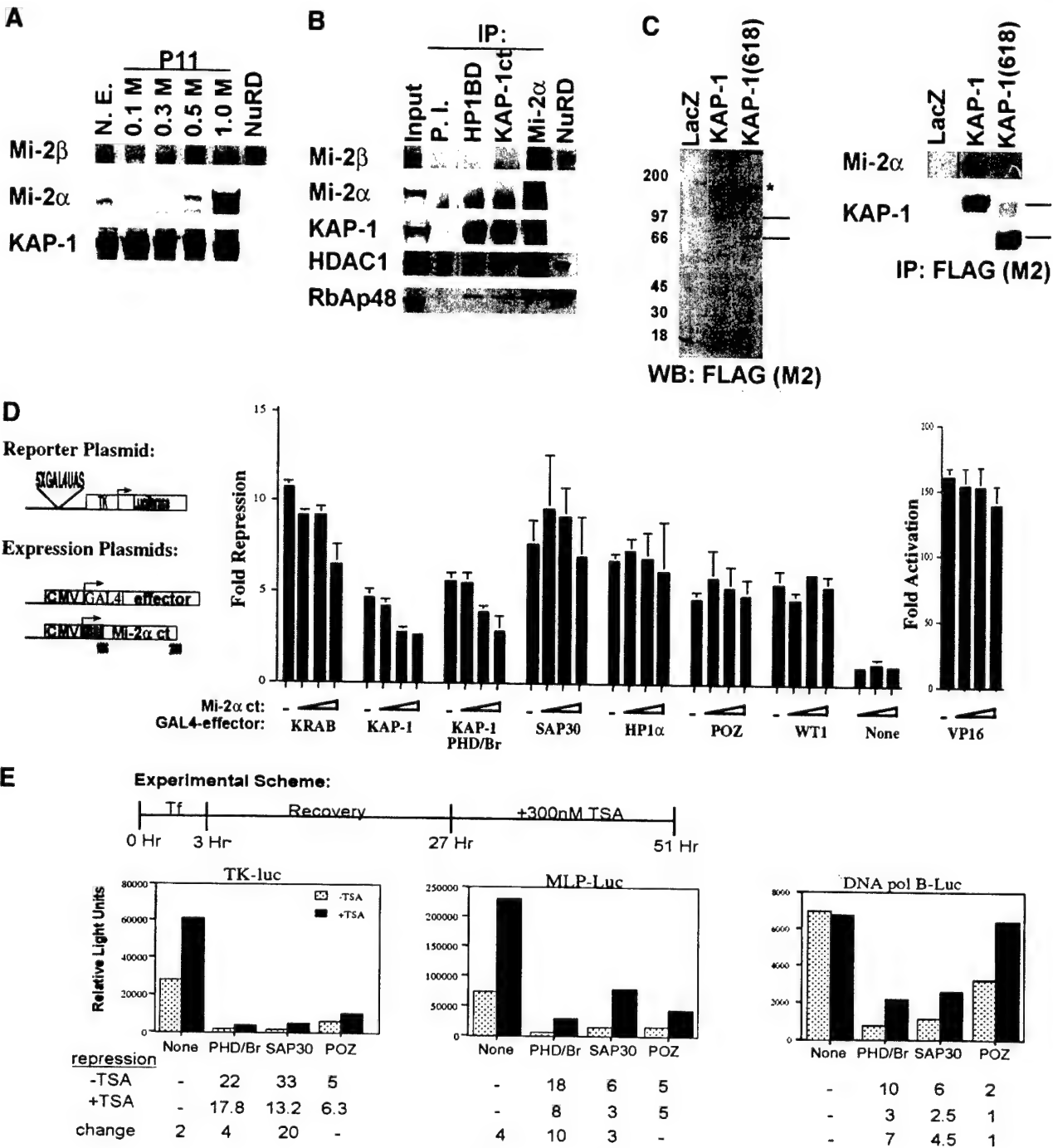
Figure 4. The PHD finger and bromodomain of KAP-1 specifically interact with the C-terminal amino acids of Mi-2 α /CHD3. (A) The yeast strain L40 cotransformed with the indicated KAP-1 mutants as LEXA DNA binding domain fusions and the rescued Mi-2 α -ct: GAL4 activation domain clone (KIP54). Positive interaction is inferred by blue yeast due to the activation of the integrated LEXA responsive *LacZ* reporter gene and subsequent hydrolysis of the synthetic substrate X-gal. (B) The interaction between KAP-1 and Mi-2 is specific for the C terminus of a unique isoform of Mi-2 α . Schematic illustration of Mi-2 α and Mi-2 β . Mi-2 α /CHD3 is a member of the CHD family of proteins which are characterized by the seven signature motifs of a "DEAH" box ATPase/Helicase. Other signature motifs include two PHD fingers (light gray hexagons), two chromodomains (black circles). Percentages represent percent identity between the two proteins at the indicated motifs. The putative KAP-1 interaction domain (KID) is indicated. In-frame fusion between the C-terminal sequences of hMi-2 α (accession no. 3298562), hCHD3 (accession no. 2645433), hMi-2 β /CHD4 (accession no. 1107696) was designed with the GAL4 activation domain. Numbers represent the corresponding amino acid in the respective sequences. L40 yeast cotransformed with the indicated GAL4 activation domain fusion proteins and the wild-type LEXA KAP-1 PHD finger and bromodomain illustrated the specificity of this interaction for Mi-2 α . (C) Amino acid sequence alignment of the C-terminal residues schematically depicted in B. Identical residues indicated in black. Boxed amino acids are unique to Mi-2 α .

of the Mi-2 α gene, which has been reported previously. Whatever the nature of this species, it does not appear to disrupt the association of KAP-1 with Mi-2 α , HDAC1 or RbAp48.

To determine the specificity of this interaction in vivo, we transiently transfected 293 cells with a Flag-tagged wild-type KAP-1 or a Flag-tagged delta PHD/bromodomain construct, KAP-1 (618). Anti-FLAG immunoprecipitates revealed the specific retention of endogenous Mi-2 α with the wild-type, full-length KAP-1

protein, but not with the delta PHD/bromodomain KAP-1 or LacZ proteins (Fig. 5C). This result demonstrates that the association of KAP-1 with Mi-2 α specifically requires sequences in the PHD finger and bromodomain.

In order to evaluate the role of Mi-2 α in KAP-1-mediated transcriptional repression, we chose to disrupt endogenous KAP-1/Mi-2 α interactions by expressing a dominant negative protein corresponding to amino acids 1686 to 2000 of Mi-2 α (Fig. 5D). Expression of these



(Legend on facing page)

amino acids dominantly inhibited heterologous KRAB-mediated repression of a DNA template in a dose-dependent manner (Fig. 5D). The same effect was observed for heterologous KAP-1-directed repression. Expression of the dominant negative protein had little effect on the basal activity of the minimal TK promoter, the activity obtained in combination with other non-KRAB related repression domain fusions, or compromised the activity of the VP16 activation domain (Fig. 5D). Because Mi-2 α has been described as an integral component of a high-molecular-weight multiprotein complex containing histone deacetylase activity, we tested whether the addition of trichostatin A (TSA) could reverse the repression activity of the KAP-1 PHD finger and bromodomain. Figure 5E illustrates that the addition of 300 nM TSA partially reversed KAP-1 PHD/bromodomain-mediated repression of three independent promoters. These data provide evidence that Mi-2 α and histone deacetylases may be one potential downstream effector of the KRAB-ZFP-KAP-1 repressor-corepressor complex in vivo.

Discussion

The recruitment of cofactors by DNA-bound transcription factors has evolved as a common, highly conserved mechanism of transcriptional regulation. Transcription factor-mediated recruitment permits gene-specific targeting of coactivator/corepressor complexes with intrinsic or associated chromatin modifying/remodeling complexes. We, and others, have previously identified a universal corepressor, KAP-1, which binds to the KRAB repression module (Friedman et al. 1996; Kim et al. 1996; Moosmann et al. 1996). Structurally, KAP-1 possesses consensus amino acid sequences for a RING finger, B-

boxes, leucine zipper coiled-coil region, PHD finger, and bromodomain (Friedman et al. 1996; Kim et al. 1996; Le Douarin et al. 1996; Moosmann et al. 1996). Biochemical studies of KAP-1 indicate that the tripartite RBCC region functions as an integrated structural unit that is necessary and sufficient for KRAB recognition and KAP-1 oligomerization (Peng et al. 2000a,b). The basis of KAP-1 in transcription repression is not fully understood, but a direct interaction with members of the HP1 protein family suggests that KAP-1 may function to repress transcription via the organization of higher order chromatin structure (Le Douarin et al. 1996; Nielsen et al. 1999; Ryan et al. 1999; Lechner et al. 2000). In this report, we present data that builds upon our understanding of the function of KAP-1 in repression of transcription. We describe a novel transcriptional repression activity defined by the PHD finger and bromodomain of KAP-1. These domains are collectively required for optimal repression of DNA templates by KAP-1, and our studies suggest that these two motifs form a functionally integrated unit, as mutations or heterologous substitutions in either domain disrupt transcriptional repression. These data are consistent with this bipartite domain providing a specific interface for protein-protein interactions. In this regard, we show evidence that the PHD finger and bromodomain of KAP-1 are collectively required to mediate an interaction with the Mi-2 α subunit of the histone deacetylase complex, NuRD, in vivo. This observation suggests that the KRAB-zinc finger protein superfamily functions as a sequence-specific targeting mechanism for the biochemical activities of the NuRD complex in vivo.

The recurrent theme that has emerged from studies aimed at understanding the function of transcriptional

Figure 5. Role of Mi-2 α and histone deacetylase in KAP-1 repression. (A) Western blot analysis of 10 μ g of phosphocellulose (P11) fractionated HeLa nuclear extracts. NuRD, conventionally purified complex as described previously (Zhang et al. 1998a). (B) In vivo association between endogenous KAP-1 and Mi-2 α . Three hundred μ g of 1.0 M P11-DEAE-bound HeLa nuclear extract was immunoprecipitated by rabbit IgG (P.I.) (lane 2), anti-KAP-1 (HP1BD/423-589) antibodies (lane 3), or anti-KAP-1 (Ct/619-835) antibodies (lane 4), or anti-Mi-2 α antibodies (lane 5). Immunoprecipitated proteins were eluted by boiling in SDS sample buffer and then separated on a 4–12% NuPAGE gradient gel (Invitrogen), followed by immunoblot analysis with antibodies against Mi-2 β , Mi-2 α , KAP-1 (RBCC), HDAC1, and RbAp48. (Input) 10 μ g of 1.0 M P11-DEAE bound extract, (NuRD) conventionally purified complex as described previously (Zhang et al. 1998a). (C) The in vivo association between KAP-1 and Mi-2 α requires the PHD finger and bromodomain of KAP-1. Expression plasmids for LacZ, FLAG-KAP-1, or FLAG-KAP-1 (618) were transfected into 293 cells. Nuclear extracts (3–5 mg) were immunoprecipitated with anti-FLAG mAb M2 (lanes 1–3). The immune complexes were separated on a 4–12% NuPAGE gradient gel (Invitrogen) followed by immunoblot analysis with anti-Mi-2 α pAb or anti-KAP-1 (RBCC/20–418) pAb. (Left panel) Anti-FLAG (M2) Western blot of transfected 293 cell nuclear extracts. Lines on right indicate the migration of full-length KAP-1 and KAP-1 (618), respectively. (Asterisk) An anti-FLAG cross-reacting species. (Right panel) The western blot analysis of anti-FLAG (M2) immunoprecipitates for Mi-2 α and KAP-1 (RBCC). Lines on the right of the KAP-1 immunoblot represent the migrations of full-length KAP-1 and KAP-1 (618), respectively. (D) Schematic diagram of the luciferase reporter and the heterologous GAL4-effector plasmid. Expression of the KAP-1 interaction domain of Mi-2 α (amino acids 1686 to 2000) dominantly inhibits heterologous KRAB- and KAP-1-mediated repression with minimal effects on heterologous repression observed for SAP30, HP1 α , BCL6-POZ, WT1 or the activation potential of VP16. (Dashes) Repression observed in the absence of transfected Mi-2 α dominant negative plasmid DNA. All experiments were done in NIH/3T3 cells with 0.1 μ g of GAL4-VP16, 0.5 μ g of GAL4-KRAB, 1 μ g of each remaining GAL4-repressor, and 0.5 μ g of the reporter plasmid. (Open triangles) Titration amounts (1 μ g, 2 μ g, and 4 μ g) of the dominant negative Mi-2 α expression plasmid transfected. Expression of all proteins was confirmed in COS1 cells (data not shown). Fold repression was calculated as described in Fig. 1. (E) Addition of the histone deacetylase inhibitor, TSA, partially reverses the repression activity of the KAP-1 PHD finger/bromodomain. NIH/3T3 cells were transiently cotransfected with 0.5 μ g of the indicated reporter plasmid and 5 μ g of the heterologous GAL4 expression plasmids KAP-1 (619 to 835), SAP30, and BCL6-POZ. Twenty-four hours posttransfection, the cells were treated with 300 nM TSA (Wako) for an additional 24 h prior to harvesting. Fold repression was calculated as described in Fig. 1. Fold repression in the absence of TSA (stippled bars); fold repression in the presence of TSA (black bars).

regulatory proteins is that highly conserved amino acid signature motifs often function as independent globular domains involved in macromolecular recognition. The identification of these signature amino acid sequence motifs and their spatial organization within a novel protein is often the first clue to its biochemical function. The PHD finger and bromodomain are two well-conserved signature motifs distributed widely among nuclear proteins with established roles in the formation, maintenance, or regulation of chromatin structure (Aasland et al. 1995; Jeanmougin et al. 1997; Winston and Allis 1999). Thus, the presence of these two motifs suggested that KAP-1 represses transcription via some chromatin-mediated mechanism. The conservation of the PHD finger and bromodomain architecturally in at least three independent families of proteins suggests that the two domains may biochemically function together (Le Douarin et al. 1995, 1996; Friedman et al. 1996; Venturini et al. 1999; Bochar et al. 2000; Jones et al. 2000a,b). In support of this hypothesis, we observed that optimal transcriptional repression by KAP-1 is dependent upon the integrity of this bipartite domain. This observation is similar to recent reports suggesting that the function of the bromodomain may require or be influenced by adjacent signature motifs (Cairns et al. 1999; Winston and Allis 1999). This intrinsic transcriptional repression activity could not be fully complemented by heterologous substitutions of either domain with related sequences. Thus, it appears from these results that the amino acid sequences of the KAP-1 PHD finger and bromodomain possess an inherent capacity to fold into an integrated functional unit, which provides a specific interface for protein-protein interactions with downstream effectors of transcriptional repression.

The PHD finger is a small domain of ~60 amino acids characteristically defined by seven cysteines and a histidine that are spatially arranged in a consensus of C4HC3 of varying lengths and composition (Aasland et al. 1995). This particular arrangement of amino acids is highly homologous to the RING finger motif, an established interface for protein-protein interactions, which is created by chelating two zinc ions in a unique cross-braced fashion (Wu et al. 1996; Borden 1998, 2000; Jensen et al. 1998). Indeed, structural analysis of the KAP-1 PHD finger indicates that two atoms of zinc are bound in a cross-braced scheme between anti-parallel β -strands (Capili et al. 2001). Like the RING finger, this finding suggests that the PHD finger functions in protein-protein interactions. Our studies represent the first detailed site-directed mutagenesis approach to correlate the molecular anatomy of this domain with a biochemical function. In general, maintenance of the zinc chelation scheme and hydrophobic amino acids that stabilize the core were essential for the structural integrity of this domain and the repression activity of KAP-1 (Capili et al. 2001). This observation was expected, as mutations of analogous amino acids in the RING finger disrupt its structure and biochemical function. Although our results clearly emphasize that the PHD finger functions in conjunction with the bromodomain, we cannot preclude autonomous

PHD finger functions. Recent studies have demonstrated that RING fingers act as E3 ubiquitin ligases through interactions with components of the ubiquitin conjugation machinery (Joazeiro and Weissman 2000). Because of the structural similarity to the RING finger motif, the PHD finger may also mediate similar functions with the ubiquitin or ubiquitin-like conjugation machinery. This hypothesis raises interesting possibilities that transcriptional repression by KAP-1 in part may result from ubiquitin mediated signaling (i.e., degradation). Alternatively, the potential for E3 ligase activity may in turn regulate the turnover or trafficking of KAP-1 itself. In any event, the experimental data from this panel of mutations will provide a basis for future studies aimed at defining new macromolecular interactions and functions for the PHD finger.

Similar to the PHD finger, the bromodomain is another conserved signature motif of ~100 amino acids that is found in 30–40 independent proteins with that function in transcriptional regulation (Jeanmougin et al. 1997). Recent structural studies of three independent bromodomains indicate that these amino acids fold into a four-helical bundle with a unique left turn topology (Dhalluin et al. 1999; Jacobson et al. 2000; Owen et al. 2000). Crystals of either the TAFII250 double bromodomain or the yGCN5p with N-terminal histone H4 peptides revealed an extensive network of interactions between the two via a hydrophobic pocket formed by the ZA and BC loops of the domain (Jacobson et al. 2000; Owen et al. 2000). Moreover, synthetically derived mutations in the bromodomain of yGCN5p were observed to impair the chromatin-related functions of this protein *in vitro* and *in vivo*, without effecting the ability of GCN5 to be assembled into SAGA (Sterner et al. 1999; Syntichaki et al. 2000). These data clearly suggest that the bromodomain is involved in modulating the activity of these transcriptional regulatory complexes. Our data from the analysis of the KAP-1 bromodomain are consistent with these hypotheses. First, comparison of the primary sequence of the KAP-1 bromodomain with pCAF, yGCN5, and TAFII250 revealed conservation of amino acids involved in stabilization of the hydrophobic core (Fig. 3B). Likewise, the amino acids that comprise the recognition site for the amide backbone of the histone tail are well conserved, confirming the likelihood that histone tails are ligands of the KAP-1 bromodomain. However, the amino acids in the ZA loop that contact the carbonyl-amide moiety of acetyl-lysines are less conserved. This deviation may represent a slightly altered specificity for the type of histone modification recognized. Second, our experimental results clearly demonstrate that the bromodomain of KAP-1 has intrinsic transcriptional repression activity, which is required for optimal KAP-1 function on DNA templates. Although the F761A, F791E, and 781trunc mutations are expected to compromise the integrity of the tertiary structure of this domain, the S730I and DD778, 781AA mutations are positioned in regions that are involved in acetyl group or histone recognition, respectively. Therefore, it is reasonable to postulate that these mutations are affecting tran-

scription without disrupting the tertiary structure of the domains. The combination of these data is consistent with the PHD finger and bromodomain functioning as a cooperative integrated unit that provides an interface for macromolecular interactions. Interestingly, the dependence of the KAP-1 interaction with Mi-2 α for the PHD finger and bromodomain is similar to recent genetic evidence that implicates an essential role for the bromodomain of GCN5 in the recruitment of the SWI/SNF chromatin remodeling activities to a targeted genomic locus in yeast (Syntichaki et al. 2000).

Macromolecular complexes with histone deacetylase and ATPase chromatin remodelling activities have clearly been implicated in chromatin dynamics and transcriptional silencing. Both Mi-2 α and Mi-2 β have been described as integral components of a multiprotein complex containing both histone deacetylase and chromatin remodeling activities (Tong et al. 1998; Wade et al. 1998; Xue et al. 1998; Zhang et al. 1998a). The association of KAP-1 with Mi-2 α builds upon our understanding of how the KRAB-ZFP-KAP-1 repressor-corepressor system mechanistically regulates gene expression. Moreover, this observation links the targeting of this multi-protein complex to specific loci via the KRAB-ZFP superfamily of repressors. We show that the KAP-1 interaction with NuRD is specifically mediated through a unique isoform of Mi-2 α . Western blot analysis of conventionally purified NuRD with antibodies specific for Mi-2 α demonstrated little immunoreactivity, a result consistent with no peptide sequences specific for Mi-2 α identified in microsequencing from previous purifications of NuRD. However, we and others can demonstrate that Mi-2 α immunoprecipitates retain HDAC1 immunoreactivity and enzymatic activity (Tong et al. 1998; Xue et al. 1998; Kim et al. 1999). These observations suggest that a Mi-2 α NuRD-like complex exists in vivo that remains to be fully defined. Consistent with this hypothesis, several NuRD preparations demonstrate heterogeneity in relation to their peptide composition (Xue et al. 1998; Kim et al. 1999).

Our data suggest that the KAP-1 corepressor utilizes a Mi-2 complex and its associated biochemical activities to repress transcription. Biochemical studies indicate that a relatively small fraction of endogenous KAP-1 stably associates with Mi-2 α in vivo, suggesting only a minor role for this interaction in KAP-1-mediated repression. From the data presented we cannot preclude other mechanisms that the PHD finger and bromodomain of KAP-1 use to repress transcription. In this regard we have characterized a direct interaction between the KAP-1 PHD finger and bromodomain and a novel protein containing a SET domain (D.C. Schultz and F.J. Rauscher III, unpubl.). Because significant evidence suggests that some feature of histones or nucleosomal structure serve as a ligand for the bromodomain, we speculate that the specific interaction between the PHD finger and bromodomain of KAP-1 with the Mi-2 α subunit of the NuRD complex may be stabilized by some interface resulting from an interaction between the bromodomain of KAP-1 and nucleosomes. This hypothesis would be con-

sistent with recent genetic evidence that indicates the bromodomain of GCN5 is required for the chromatin remodeling activities of the SWI/SNF complex at a genomic locus in yeast (Syntichaki et al. 2000). In this regard, both the *His3* and the *LacZ* reporters used in the selection process for positive interacting clones in the two-hybrid assay were chromosomally integrated. Thus, it is reasonable to hypothesize that the stability of this interaction is disrupted during the extraction process. The role of Mi-2 associated histone deacetylase activity in KRAB-KAP-1 mediated repression will require the analysis of histone acetylation patterns within regulatory elements of single copy KRAB-ZFP target genes.

The interaction between KAP-1 and Mi-2 α is one potential mechanism by which the biochemical activities of the NuRD complexes can be targeted in a sequence-specific manner to genes in vivo by virtue of the selectivity conferred by the C2H2 DNA binding domain of the KRAB-ZFPs. Consistent with this hypothesis, the lymphoid-specific, sequence-specific transcription factor Ikaros stably associates with the NuRD complex (Kim et al. 1999). Immuno-FISH experiments revealed that repressed genes by Ikaros were colocalized with components of NuRD and the HP1 protein in nuclear territories juxtaposed to centromeric heterochromatin during B-cell maturation (Brown et al. 1997). Similarly, we and others have reported a direct biochemical interaction between KAP-1 and mammalian homologs of the HP1 protein family, and have shown that KAP-1 and mammalian HP1 proteins can physically occupy the same spatial domains in interphase nuclei of NIH/3T3 cells (Ryan et al. 1999). The combination of these findings implies a potential link between multi-protein complexes that promote transcriptional repression by histone deacetylation and repression mediated by organization of high-order chromatin structure. In support of this hypothesis, SAP30, a novel member of the SIN3 histone deacetylase complex, has been observed to effect telomere silencing in yeast (Zhang et al. 1998b) and genetic screens for dominant suppressors of position-effect-variegation in *Drosophila melanogaster* have identified mutant alleles of *HDAC1* (Mottus et al. 2000). Furthermore, genetic analyses in *D. melanogaster* demonstrate an epistatic relationship between dMi-2 and polycomb silencing of homeotic genes (Kehle et al. 1998). This apparent coupling of two independent repression mechanisms may lead to synergistic repression of their apparent target genes and promote epigenetic gene silencing.

The estimated 300 to 700 KRAB zinc finger proteins in the human proteome potentially make this superfamily of transcriptional repressors one of the master regulators of sequence-specific gene silencing in vertebrates. Transcriptional repression by a KRAB domain requires interaction with the corepressor, KAP-1. Thus, it appears that the effector molecules of KRAB-mediated transcriptional repression are likely due to a network of protein interactions with KAP-1. We suggest that the KAP-1 corepressor may function to repress transcription through several distinct mechanisms, including histone deacetylation and heterochromatinization. Unlike prokaryotic genes,

eukaryotic genes exist in a restrictive state by virtue of nucleosome-based chromatin structure [Struhl 1999]. In this model, the role of sequence-specific repressors like the superfamily of KRAB-ZFPs may be to create a silent gene environment completely resistant to activator and/or core transcription machinery binding. We propose that KRAB zinc finger proteins selectively bind promoter sequences and recruit the KAP-1 corepressor to the targeted locus. This complex in turn recruits the NuRD complex, which deacetylates histones in the promoter region, creating a favorable condition for HP1 proteins to nucleate a local heterochromatin environment that results in effective gene silencing. The abundance of KRAB domain proteins and the diverse capacity of the KAP-1 corepressor to repress transcription through multiple independent mechanisms emphasizes the potential of the KRAB-KAP-1 repressor-corepressor system to function as a master regulator of transcriptional gene silencing.

Materials and methods

Plasmid construction

The pM2-KAP-1 (293-835) mammalian expression vector has been described previously [Friedman et al. 1996]. The GAL4-KAP-1 fusion proteins including 408-835, 408-618, 478-835, 478-680, and 478-618 were created by PCR using the primers 408for (*Bam*HI) 5'-CCGGGATCCAGATTGTGGCAGAGC GTCCTG-3', 478for (*Bam*HI) 5'-CCGGGATCCAGGTGAGC GGCTTATGCGC-3', 618rev (*Hind*III) 5'-GATAAGCTTTC ACGGGCCACCACTGGGGC-3', 835rev (*Xba*I) 5'-GCTAT CTAGACTAAATGGTGGCACTGTCATCCAGG-3', respectively. The resulting PCR products were digested with the restriction endonucleases indicated in brackets preceding the primer sequence and cloned into the corresponding restriction sites of the vector pM1. The GAL4-KAP-1 (619-835) expression plasmid was constructed by subcloning an *Xba*I fragment from pBL-KAP-1 into the *Xba*I site of pM1. The GAL4-KAP-1 (619-679) expression plasmid was created by PCR using the primers 619for (*Eco*RI) 5'-TAGCGAATTCGGAACCCTGGATGACAG TGC-3' and 679rev (*Sal*I) 5'-ATCGGTGGACATCCTCCTCC TTCAGGTCAG-3'. The resulting PCR product was digested with the restriction endonucleases indicated in brackets and cloned into the corresponding sites of the vector pM1. C-terminal to codon 679 of KAP-1 the protein reads VDASAEASR*. The GAL4-KAP-1 (674-835) expression plasmid was created by PCR, using the primers 674for (*Bam*HI) 5'-ATCGGATCCGAC CTGAAGGAGGAGGATGCG-3' and 835rev (*Hind*III) 5'-GAT CCCGGAAGCTTTCAGGGGCCATCACCTGG-3'. The resulting PCR product was digested with the restriction endonucleases indicated in brackets and cloned into the corresponding sites of the vector pM2.

Site-directed point mutations in the KAP-1 PHD finger and bromodomain were engineered by standard overlap extension PCR-mediated mutagenesis procedures. The mutagenic primers for the described mutations contained the following codons: C628R, TGC to CGC; V630S, GTC to AGC; C651F, TGT to TTT; P654C, CCG to TGC; Q657E, CAG to GAG; E715K, GAA to AAA; S730I, TCC to ATC; L742T, CTG to ACG; F761A, TTT to GCT; D778, 781AA, GAC to GCT; F791E, TTC to GAG. The pM1-KAP-1 (619-835) trunc781 resulted from spontaneous deletion of G2444 from the nucleotide sequence during PCR of the DD 778,781 AA mutation and a reading frame shift that results

in a protein with 15 novel amino acids, AKAACSPSSGCSA SSRRA* starting at codon 778 of KAP-1.

The GAL4 expression plasmids for the PHD finger and bromodomain of TIF1 α (amino acids 785-1016), TIF1 γ (amino acids 881-1120), and WCRF180 (amino acids 1141-1556) were created by PCR using the primers TIF1 α for (*Sma*I) 5'-GATCCCGGGAGGAAGGAGGATGACCCC-3', TIF1 α rev (*Sma*I) 5'-GTACCCGGGTACTTAAGCAGCTGGCG-3', TIF1 γ for (*Sma*I) 5'-GATCCCGGGAATAAAGATGATGACCCC-3', TIF1 γ rev (*Sma*I) 5'-CTGCCCCGGTCTGACTTTAGGCGT-3', WCRF180for (*Sma*I) 5'-GATCCCGGGATATGGTCTAAATC TATA-3', and WCRF180rev (*Sma*I) 5'-CTGCCCCGGTCAGAT TCGTGACTTTTTTGC-3', respectively. The resulting PCR products were digested with the restriction endonucleases indicated in brackets and cloned into the corresponding sites of either pM1 or pM3. The PHD finger and bromodomain chimeras were engineered by standard overlap extension PCR-mediated mutagenesis procedures. Forward primers were: ATRX 5' (*Sma*I) 5'-GACCCGGGTAGCCGTGACTCAGATGG-3', Mi-2 α 5' (*Sma*I) 5'-GATCCCGGGATGGCTACGAGACGGATC-3', and as indicated above for WCRF180 and KAP-1. Fusion primers were as follows: TIF1 α /KAP-1for 5'-AAGCCAGAGGTTGAC TATGGCAGCTCAGCCTGGAT-3', TIF1 γ /KAP-1for 5'-AAG CCAGAAGTTGAATATGGCAGCCTCAGCCTGGAT-3', AT RX/KAP-1for 5'-ACTGCATGTAACAGCGTAGGCAGCCTCA GCCTCAGC-3', Mi-2 α /KAP-1for 5'-GTCCAGTGGGAGG CCAAGGGCAGCCTCAGCCTCAGC-3', WCRF180/KAP-1for 5'-CGTTCTAGAAGACTCTCCGGCAGCCTCAGCCTCAGC 3', KAP-1/TIF1 α for 5'-GACCTGAAGGAGGAGGATGATTGT GATGTTCCAGT-3', KAP-1/TIF1 γ for 5'-GACCTGAAGGA GGAGGATGATTGTGATAATTTGCAA-3', KAP-1/WCRF180for 5'-GACCTGAAGGAGGAGGATAGATCTGTAAATATTGCT-3', KAP-1/GCN5for 5'-GACCTGAAGGAGGAGGATACAGGC TGGAAGCCATTG-3'. Reverse primers were as follows: GCN5rev (*Sma*I) 5'-GATCCCGGGCTACTTGTCAATGAGGCC-3', and as indicated above for KAP-1, TIF1 α , TIF1 γ , and WCRF180. Second-round PCR products were digested with restriction endonucleases as defined in the parenthesis preceding the primer sequence and cloned into the corresponding sites of either pM1 or pM3.

The LEXA-KAP-1 (amino acids 619-835) yeast expression plasmid was generated by the ligation of an *Xba*I fragment from pBL-KAP-1 into pBTM116. PHD finger and bromodomain mutants were subcloned from pM1 to pBTM116 as a *Sma*I fragment. GAL4 activation domain fusions with the C terminus of Mi-2 α delta ct and Mi-2 β were created by PCR using the following primers, respectively: Mi-2 α for (*Bam*HI) 5'-GTAACGGA TCCAGGGCCTCGAGATGAGCCAC3', Mi-2 α delta ct (*Bam*HI) 5'-GATCGGATCCTCAGCTTGGTGGCGGCTGTGATGAAG-3', Mi-2 β for (*Bam*HI) 5'-GTAACGGATCCAAATGGAGAGAC CCCAAGGACCTG-3', Mi-2 β rev (*Bam*HI) 5'-GATCGGATC CTCACTGCTGGGCTACCTGCTG-3'. Resultant PCR products were digested with *Bam*HI and cloned into the corresponding restriction site of pACTII (Clontech).

The CMV-Mi-2 α ct mammalian expression plasmid was created by subcloning a *Bam*HI/*Bam*HI DNA fragment encoding all amino acids of the rescued cDNA in-frame with an N-terminal 6His-tag possessing a consensus Kozak sequence and initiator methionine in pcDNA3.1 (Invitrogen). The GAL4-KRAB, GAL4-HP1 α , and GAL4-WT1 expression plasmids have been described previously [Margolin et al. 1994; Lechner et al. 2000]. The GAL4-SAP30 expression plasmid was kindly provided by Danny Reinberg (Howard Hughes Medical Institute, UMDNJ, NJ). The GAL4-BCL6-POZ expression plasmid was kindly provided by Vivian Bardwell (University of Minnesota, Minneapolis).

The FLAG-KAP-1 expression plasmid was constructed with a consensus Kozak sequence, an initiator methionine, and a FLAG-tag (DTKDDDDK) followed by amino acids 20–835 of KAP-1 in the *Bam*HI/*Xba*I restriction sites of pcDNA3 (Invitrogen). Similarly, the FLAG-KAP-1 (618) expression plasmid was constructed encoding amino acids 20–618 of KAP-1 in the *Bam*HI/*Xba*I restriction sites of pcDNA3 (Invitrogen).

Appropriate reading frame fusions and integrity of flanking sequences for all constructs created by PCR was confirmed by DNA sequence analysis of both strands.

Yeast two-hybrid system

The yeast two-hybrid system as modified by Stan Hollenberg was used for all yeast experiments. A human oligo-dT-primed B-cell cDNA library was screened as described previously [Jensen et al. 1998].

Transient transfection

Protein expression from all plasmids was confirmed by transient transfection of COS-1 cells followed by immunoprecipitation of [³⁵S]methionine labeled cell extracts with anti-GAL4(DBD) IgG sc-577 (Santa Cruz), as described previously. Preparation of nuclear extracts from transfected COS1 cells was described previously. Electrophoretic mobility shift assays were done as described previously. All luciferase assays from transient transfections were done as described previously [Ryan et al. 1999].

Immunoprecipitations

Affinity-purified polyclonal antibodies raised against amino acids 20–418 (RBCC), 423–589 (HP1BD), and 618–835 (Ct) of KAP-1 and amino acids 1515 to 1708 of Mi-2 α (accession no. 3298562) were coupled to protein A-agarose (Repligen) by dimethylpimelidate as described previously [Harlow and Lane 1988]. Preparation of HeLa cell nuclear extract and phosphocellulose (P11) fractionation was done as described previously [Dignam et al. 1983]. Three hundred micrograms of DEAE-bound 1.0 M P11 fractions was incubated with 50 μ g of protein A-coupled antibody at 4°C for 4 h. Immune complexes were washed 3 times with BC500 (20 mM Tris-HCl at pH 8.0, 500 mM KCl, 0.2 mM EDTA, 10 % glycerol, 0.2 mM PMSF, 0.1% NP-40), once with BC100 (20 mM Tris-HCl at pH 8.0, 100 mM KCl, 0.2 mM EDTA, 10 % glycerol, 0.2 mM PMSF, 0.1% NP-40), and eluted with 0.1 M glycine (pH 2.7). Eluted proteins were resolved on a 4–12% NuPAGE gel in MOPS running buffer (Invitrogen), then transferred to PVDF, and antigens were detected as described previously [Ryan et al. 1999]. Nuclear extracts from transiently transfected 293 cells were prepared as described previously [Ryan et al. 1999]. Three to five milligrams of nuclear extract adjusted to 100 mM NaCl were incubated with 100 μ g of anti-FLAG M2 (Sigma) at 4°C for 4 h. Bound immune complexes were washed, eluted and analyzed as described. Antibodies specific for Mi-2 β (CHD4-N) were kindly provided by W. Wong (National Institute on Aging, Baltimore, MD). Antibodies against HDAC1 were kindly provided by D. Reinberg. RbAp48 was detected with mAb 13D10 (GeneTex).

Acknowledgments

D.C.S. was supported in part by the Wistar Basic Cancer Research Training Grant CA 09171 and DAMD 17-98-1-8269 grant. We acknowledge Robert F. Ryan for doing the original

two-hybrid screen. We thank Trevor Woodage for Mi-2 α cDNA sequences, David Picketts for the hATRX PHD finger sequence, and Shelley Berger for hGCN5 bromodomain sequences. F.J.R. is supported in part by National Institutes of Health grants CA 52009, Core grant CA 10815, DK 49210, GM 54220, DAMD 17-96-1-6141, ACS NP-954, the Irving A. Hansen Memorial Foundation, the Mary A. Rumsey Memorial Foundation, and the Pew Scholars Program in the Biomedical Sciences.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Aasland, R., Gibson, T.J., and Stewart, A.F. 1995. The PHD finger: Implications for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.* **20**: 56–59.
- Agata, Y., Matsuda, E., and Shimizu, A. 1999. Two novel Kruppel-associated box-containing zinc-finger proteins, KRAZ1 and KRAZ2, repress transcription through functional interaction with the corepressor KAP-1 (TIF1 β /KRIP-1). *J. Biol. Chem.* **274**: 16412–16422.
- Bellefroid, E.J., Poncellet, D.A., Lecocq, P.J., Revelant, O., and Martial, J.A. 1991. The evolutionarily conserved Kruppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. *Proc. Natl. Acad. Sci.* **88**: 3608–3612.
- Bjorses, P., Halonen, M., Palvimo, J.J., Kolmer, M., Aaltonen, J., Ellonen, P., Perheentupa, J., Ulmanen, I., and Peltonen, L. 2000. Mutations in the AIRE gene: Effects on subcellular location and transactivation function of the autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy protein. *Am. J. Hum. Genet.* **66**: 378–392.
- Bochar, D.A., Savard, J., Wang, W., Lafleur, D.W., Moore, P., Cote, J., and Shiekhatter, R. 2000. A family of chromatin remodeling factors related to Williams syndrome transcription factor. *Proc. Natl. Acad. Sci.* **97**: 1038–1043.
- Borden, K.L.B. 1998. RING fingers and B-boxes: Zinc-binding protein–protein interaction domains. *Biochem. Cell Biol.* **76**: 351–358.
- Borden, K.L.B. 2000. RING Domains: Master builders of molecular scaffolds? *J. Mol. Biol.* **295**: 1103–1112.
- Brown, K.E., Guest, S.S., Smale, S.T., Hahm, K., Merken-schlager, M., and Fisher, A.G. 1997. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**: 845–854.
- Cairns, B.R., Schlichter, A., Erdjument-Bromage, H., Tempst, P., Kornberg, R.D., and Winston, F. 1999. Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH, and bromodomains. *Mol. Cell* **4**: 715–723.
- Capili, A.D., Schultz, D.C., Rauscher III, F.J., and Borden, K.L.B. 2001. Solution structure of the PHD domain from the KAP-1 corepressor: Structural determinants for PHD, RING, and LIM zinc binding domains. *EMBO J.* **20**: 165–177.
- Delmas, V., Stokes, D.G., and Perry, R.P. 1993. A mammalian DNA-binding protein that contains a chromodomain and an SNF2/SWI2-like helicase domain. *Proc. Natl. Acad. Sci.* **90**: 2414–2418.
- Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K., and Zhou, M.M. 1999. Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**: 491–496.
- Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*

- 11: 1475-1489.
- Friedman, J.R., Fredericks, W.J., Jensen, D.E., Speicher, D.W., Huang, X.P., Neilson, E.G., and Rauscher III, F.J. 1996. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes & Dev.* **10**: 2067-2078.
- Gibbons, R.J., Bachoo, S., Picketts, D.J., Aftimos, S., Ascnbauer, B., Bergoffen, J., Berry, S.A., Dahl, N., Fryer, A., Keppler, K., et al. 1997. Mutations in transcriptional regulator ATRX establish the functional significance of a PHD-like domain. *Nat. Genet.* **17**: 146-148.
- Hampsey, M. and Reinberg, D. 1999. RNA polymerase II as a control panel for multiple coactivator complexes. *Curr. Opin. Genet. Dev.* **9**: 132-139.
- Harlow, E. and Lane, D. 1988. *Antibodies: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Jacobson, R.H., Ladurner, A.G., King, D.S., and Tjian, R. 2000. Structure and function of a human TAFII250 double bromodomain module. *Science* **288**: 1422-1425.
- Jeanmougin, F., Wurtz, J.M., Le Douarin, B., Chambon, P., and Losson, R. 1997. The bromodomain revisited. *Trends Biochem. Sci.* **22**: 151-153.
- Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y., et al. 1998. BAP1: A novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* **16**: 1097-1112.
- Joazeiro, C.A. and Weissman, A.M. 2000. RING finger proteins: Mediators of ubiquitin ligase activity. *Cell* **102**: 549-552.
- Jones, M.H., Hamana, N., Nezu, J., and Shimane, M. 2000a. A novel family of bromodomain genes. *Genomics* **63**: 40-45.
- Jones, M.H., Hamana, N., and Shimane, M. 2000b. Identification and characterization of BPTF, a novel bromodomain transcription factor. *Genomics* **63**: 35-39.
- Kehle, J., Beuchle, D., Treuheit, S., Christen, B., Kennison, J.A., Bienz, M., and Muller, J. 1998. dMi-2, a hunchback-interacting protein that functions in polycomb repression. *Science* **282**: 1897-1900.
- Kim, J., Sif, S., Jones, B., Jackson, A., Koipally, J., Heller, E., Winandy, S., Viel, A., Sawyer, A., Ikeda, T., et al. 1999. Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. *Immunity* **10**: 345-355.
- Kim, S.S., Chen, Y.M., O'Leary, E., Witzgall, R., Vidal, M., and Bonventre, J.V. 1996. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc. Natl. Acad. Sci.* **93**: 15299-15304.
- Le Douarin, B., Zechel, C., Garnier, J.M., Lutz, Y., Tora, L., Pierrat, P., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. 1995. The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.* **14**: 2020-2033.
- Le Douarin, B., Nielsen, A.L., Garnier, J.M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. 1996. A possible involvement of TIF1 α and TIF1 β in the epigenetic control of transcription by nuclear receptors. *EMBO J.* **15**: 6701-6715.
- Lechner, M.S., Begg, G.E., Speicher, D.W., and Rauscher III, F.J. 2000. Molecular determinants for targeting HPI-mediated gene silencing: A direct chromoshadow domain-KAP-1 corepressor interaction is essential. *Mol. Cell. Biol.* **20**: 6449-6465.
- Margolin, J.F., Friedman, J.R., Meyer, W.K., Vissing, H., Thiesen, H.J., and Rauscher III, F.J. 1994. Kruppel-associated boxes are potent transcriptional repression domains. *Proc. Natl. Acad. Sci.* **91**: 4509-4513.
- Moosmann, P., Georgiev, O., Le Douarin, B., Bourquin, J.P., and Schaffner, W. 1996. Transcriptional repression by RING finger protein TIF1 beta that interacts with the KRAB repressor domain of KOX1. *Nucleic Acids Res.* **24**: 4859-4867.
- Moosmann, P., Georgiev, O., Thiesen, H.J., Hagmann, M., and Schaffner, W. 1997. Silencing of RNA polymerases II and III-dependent transcription by the KRAB protein domain of KOX1, a Kruppel-type zinc finger factor. *Biol. Chem.* **378**: 669-677.
- Mottus, R., Sobel, R.E., and Grigliatti, T.A. 2000. Mutational analysis of a histone deacetylase in *Drosophila melanogaster*: Missense mutations suppress gene silencing associated with position effect variegation. *Genetics* **154**: 657-668.
- Nielsen, A.L., Ortiz, J.A., You, J., Oulad-Abdelghani, M., Khechumian, R., Gansmuller, A., Chambon, P., and Losson, R. 1999. Interaction with members of the heterochromatin protein 1 (HPI) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *EMBO J.* **18**: 6385-6395.
- Orphanides, G., Lagrange, T., and Reinberg, D. 1996. The general transcription factors of RNA polymerase II. *Genes & Dev.* **10**: 2657-2683.
- Owen, D.J., Ornaghi, P., Yang, J.-C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P., and Travers, A.A. 2000. The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase Gcn5p. *EMBO J.* **19**: 6141-6149.
- Peng, H., Begg, G.E., Harper, S.L., Friedman, J.R., Speicher, D.W., and Rauscher III, F.J. 2000a. Biochemical analysis of the KRAB transcriptional repression domain: Spectral, kinetic and stoichiometric properties of the KRA-KAP-1 complex. *J. Biol. Chem.* **275**: 18000-18010.
- Peng, H., Begg, G.E., Schultz, D.C., Friedman, J.R., Jensen, D.E., Speicher, D.W., and Rauscher III, F.J. 2000b. Reconstitution of the KRAB-KAP-1 repressor complex: A model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. *J. Mol. Biol.* **295**: 1139-1162.
- Ryan, R.F., Schultz, D.C., Ayyanathan, K., Singh, P.B., Friedman, J.R., Fredericks, W.J., and Rauscher III, F.J. 1999. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HPI proteins: A potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell. Biol.* **19**: 4366-4378.
- Sternier, D.E., Grant, P.A., Roberts, S.M., Duggan, L.J., Belotserkovskaya, R., Pacella, L.A., Winston, F., Workman, J.L., and Berger, S.L. 1999. Functional organization of the yeast SAGA complex: Distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. *Mol. Cell. Biol.* **19**: 86-98.
- Strahl, B.D. and Allis, C.D. 2000. The language of covalent histone modifications. *Nature* **403**: 41-45.
- Struhl, K. 1999. Fundamentally different logic of gene regulation in eukaryotes and prokaryotes. *Cell* **98**: 1-4.
- Syntichaki, P., Topalidou, I., and Thireos, G. 2000. The Gcn5 bromodomain co-ordinates nucleosome remodelling. *Nature* **404**: 414-417.
- Tong, J.K., Hassig, C.A., Schnitzler, G.R., Kingston, R.E., and Schreiber, S.L. 1998. Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature* **395**: 917-921.
- Tyler, J.K. and Kadonaga, J.T. 1999. The "dark side" of chromatin remodeling: Repressive effects on transcription. *Cell*

- 99: 443-446.
- Venturini, L., You, J., Stadler, M., Galien, R., Lallemand, V., Koken, M.H., Mattei, M.G., Ganser, A., Chambon, P., Losson, R., and de The, H. 1999. TIF1 γ , a novel member of the transcriptional intermediary factor 1 family. *Oncogene* **18**: 1209-1217.
- Wade, P.A., Jones, P.L., Vermaak, D., and Wolffe, A.P. 1998. A multiple subunit Mi-2 histone deacetylase from *Xenopus laevis* cofractionates with an associated Snf2 superfamily ATPase. *Curr. Biol.* **8**: 843-846.
- Winston, F. and Allis, C.D. 1999. The bromodomain: A chromatin-targeting module? *Nat. Struct. Biol.* **6**: 601-604.
- Woodage, T., Basrai, M.A., Baxevasis, A.D., Hieter, P., and Collins, F.S. 1997. Characterization of the CHD family of proteins. *Proc. Natl. Acad. Sci.* **94**: 11472-11477.
- Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C., Hwang, L.Y., Bowcock, A.M., and Baer, R. 1996. Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nat. Genet.* **14**: 430-440.
- Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J., and Wang, W. 1998. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol. Cell* **2**: 851-861.
- Zhang, Y., LeRoy, G., Seelig, H.P., Lane, W.S., and Reinberg, D. 1998a. The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell* **95**: 279-289.
- Zhang, Y., Sun, Z.W., Iratni, R., Erdjument-Bromage, H., Tempst, P., Hampsey, M., and Reinberg, D. 1998b. SAP30, a novel protein conserved between human and yeast, is a component of a histone deacetylase complex. *Mol. Cell* **1**: 1021-1031.

**The KRAB-ZFP:KAP-1 Repression System
Recruits the Histone Deacetylase Complex, NuRD, via an
Interaction Between the PHD and Bromodomains of KAP1
and the Mi-2 Subunit.**

David C. Schultz¹, Allan D. Capili², Katherine L. B. Borden²,
and Frank J. Rauscher III¹. ¹The Wistar Institute, Philadelphia
PA. ²Mount Sinai School of Medicine, New York, NY.

The PHD finger and bromodomain are two highly conserved protein motifs found in proteins with transcriptional regulatory functions. Naturally occurring mutations in the PHD finger contribute to a variety of human diseases including ATRX syndrome, myeloid leukemias, and autoimmune dysfunction. Optimal transcriptional repression by KAP-1, a universal corepressor for the KRAB-ZFP superfamily of transcriptional repressors, requires an intact PHD finger and bromodomain at its COOH-terminus. Site-directed mutations in the KAP-1 PHD finger or the bromodomain specifically disrupt the repression function of this bi-partite domain. We have determined a solution structure for the PHD finger of KAP-1. Our studies reveal that the PHD domain binds zinc in a cross-brace topology reminiscent of RING domains. Mechanistically, the KAP-1 PHD and bromodomains are required to mediate an interaction with the Mi-2 α subunit of the NuRD *in vivo* as determined by co-immunoprecipitation. These data indicate that the PHD finger and bromodomain provide an interface for protein-protein interactions. Moreover, the data suggest the KRAB-ZFP:KAP-1 repression complex functions to target the histone deacetylase activity of the NuRD complex to specific gene promoters *in vivo*. This work was funded in part by DAMD 17-98-1-8269 to DCS.

SETDB1: A novel SET domain protein that contains histone H3-lysine 9 specific methylase activity, recruits HP1 to endogenous, euchromatic targets.

David C. Schultz^{1*}, Kasirajan Ayyanathan¹, Frank J. Rauscher, III¹. *Department of Pharmacology, Case Western Reserve University, Cleveland, OH. ¹The Wistar Institute, Philadelphia, PA.

The 220 KRAB domain-zinc-finger proteins encoded by the human genome function as gene-specific silencers. Silencing requires binding to the co-repressor, KAP-1 that in turn coordinates the activities of large macromolecular complexes that modify chromatin structure. The PHD finger and bromodomain of KAP-1 recruits the NuRD HDAC complex whereas a separate region of KAP-1 binds directly to the chromoshadow domain of the HP1 protein family. Since the HP1 chromodomain can bind to the methylated Lys-9 in histone H3, we looked for this activity in KAP-1 repression complexes. Here we report the discovery of a novel KAP-1 associated H3, Lys-9 specific histone methyltransferase, SETDB1. The enzymatic activity of SETDB1 increased HP1 binding to histone H3 and CHIP experiments showed colocalization of KAP-1, SETDB1, and HP1 along with increased H3 Lys-9 methylation of chromatin at an endogenous target gene to stably repress gene expression. KAP-1 is the first example of a co-repressor/scaffold protein that can coordinate the sequential recruitment of HDAC complexes, histone methylase, and the deposition of HP1 at a euchromatic locus to silence gene expression in a manner consistent with epigenetic regulation. This work was funded in part by DAMD 17-98-1-8269 to DCS

David C. Schultz, Ph.D
(215) 898-0903
J5
Poster Session 3

SETDB1: A new SET domain protein with histone H3, lysine-9-specific methyltransferase activity that contributes to silencing endogenous, euchromatic genes by recruiting HP1. David C. Schultz^{1*}, Kasirajan Ayyanathan¹, Frank J. Rauscher, III¹. *Department of Pharmacology, Case Western Reserve University, Cleveland, OH. ¹The Wistar Institute, Philadelphia, PA.

Macromolecular protein complexes containing enzymatic activity that modifies the NH₂-terminal tails of the core histones have emerged as key regulators of gene regulation in eukaryotes. Oncogenic transformation frequently results in abrogation of this regulatory machinery leading to global alterations in chromatin structure and gene expression that contribute to malignant progression. The BRCA1-associated protein ZBRK1 is a member of the KRAB domain-zinc-finger protein (ZFPs) superfamily of gene-specific silencers. Gene silencing by ZBRK1 requires binding to the co-repressor, KAP1, that in turn coordinates the activities of large macromolecular complexes that modify chromatin structure. The PHD finger and bromodomain of KAP1 recruits the NuRD histone deacetylase complex whereas a separate region of KAP1 binds directly to the chromoshadow domain of the heterochromatin protein-1 (HP1) family. Recently it has been discovered that the chromodomain of HP1 binds to methylated Lys-9 (K9) in the NH₂-terminus of histone H3. Since KAP1 may coordinate HP1 deposition and histone methylation, we looked for this enzymatic activity in endogenous KAP1 complexes. Here we report the discovery and cloning of a novel KAP1 associated histone H3-K9 specific methyltransferase, SETDB1. Mutations of evolutionarily conserved amino acid residues in the catalytic domain, including the pre-SET, SET and post-SET domains, significantly impairs the enzymatic activity of SETDB1. *In vitro* methylation of the NH₂-terminal tails of histone H3 by SETDB1 enhances the binding of HP1 proteins to this histone. Intact chromo- and chromoshadow domains in HP1 are required for this binding. Surprisingly, SETDB1 is localized predominantly to euchromatic regions of the nucleus in interphase NIH3T3 cells and overlaps with histone H3-K9 methylation in non-pericentromeric regions of chromatin. Chromatin immunoprecipitation experiments reveal the co-localization of KAP-1, SETDB1, HP1 and enriched histone H3 K9 methylation in chromatin at an endogenous, euchromatic gene regulated by the KRAB:KAP1:HP1 repression system. KAP-1 is the first example of a co-repressor protein that can coordinate the sequential recruitment of histone deacetylases, methylases, and the deposition of HP1 at a euchromatic locus to silence gene expression. Since these studies define the machinery utilized by ZBRK1 to regulate gene expression, current studies are focused at evaluating the role of KAP1:SETDB1:HP1 and ZBRK1:BRCA1 regulation of endogenous target genes such as Gadd45 and WAF1:p21 in breast tumors.